SCREENING OF CYP450 2C9 GENETIC POLYMORPHISM ASSOCIATED WITH ACENOCOUMAROL SENSITIVITY IN DVT CASES

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By

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CERTIFICATE

This is to certify that this dissertation entitled **"SCREENING OF CYP450 2C9 GENETIC POLYMORPHISM ASSOCIATED WITH ACENOCOUMAROL SENSITIVITY IN DVT CASES"** submitted by **Dr.M.SELVI** appearing for M.D. Branch I-GeneralMedicine Degree examination in April,2014is a bonafide record of work done by here under my direct guidance and supervision in partial fulfillment of regulations of the TamilNadu Dr. M.G.R.Medical University, Chennai. I forward this to the TamilNadu Dr.M.G.R.Medical University, Chennai, Tamil Nadu, India.

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DECLARATION

I solemnly declare that the dissertation entitled **"SCREENING OF CYP450 2C9 GENETIC POLYMORPHISM ASSOCIATED WITH ACENOCOUMAROL SENSITIVITY IN DVT CASES"** is done by me at Madras Medical College & Rajiv Gandhi Government General Hospital, Chennai during June 2013 to Dec 2013 under the guidance and supervision of **Prof K.SIVASUBRAMANIAN, M.D.** The dissertation is submitted to The Tamil Nadu Dr.M.G.R. Medical University towards the partial fulfillment of requirements for the award of M.D. Degree (Branch-I) in General Medicine.

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CONTENTS

Screening of CYP450 2C9 genetic polymorphism associated with Acenocoumarol sensitivity in DVT cases

Abstract

Background: Deep vein thrombosis is a life threatening disease and remains one of the leading causes of morbidity and mortality. Warfarin/Acenocoumarol are coumarin derivatives widely used as an anticoagulant for treating DVT patients with a narrow therapeutic index and wide inter – individual variability in dose requirement due to the genetic variation in CYP450 2C9 and VKORC1 and associated with the risk of thromboembolism or bleeding depending on underdosing or overdosing respectively.

Aim: To screen the CYP450 2C9*2,*3,*4 and *5 genotypes associated with Acenocoumarol sensitivity in Indian DVT cases.

Methods: Fifty cases with clinical evidence of DVT were selected after confirmed with Doppler study of affected limb from the inpatients of Madras Medical College and Rajiv Gandhi Government General hospital. Blood samples were collected and DNA was isolated from the blood by phenol chloroform extraction method. PCR amplification of CYP450 2C9 exon 1 and exon 4 were amplified using respective sequence specific primer pairs. PCR products were analysed by PCR-RFLP and direct sequencing.

Conclusion: CYP450 2C9 genetic variants *2, *3, *4 and *5 were analyzed in 42 cases successfully. Out of 42 samples analyzed 5 samples were found to be heterozygous for CYP4502C9 *2 variant. All other cases were found to carry wild type alleles. The cases having CYP450 2C9*2 heterozygous variant require decreased dose of warfarin by approximately 20-30% compared to CYP2C9 wild type individuals.

Key words: DVT, WARFARIN, CYP450 2C9.

INTRODUCTION

Deep vein thrombosis is clotting of blood within the deep venous system such as femoral vein, popliteal vein and one of the leading causes of hospital morbidity and mortality. Lower extremity DVT may leads to pulmonary embolism, post thrombotic syndrome, paradoxical embolization or phlegmasia cerulae dolens which can result in major disability or death. Many Studies have shown that patients having chronic venous obstruction result in post thrombotic syndrome and that multi segment venous involvement and Ileofemoral obstruction lead to most profound morbidity(1) (2).

Oral anticoagulants like Warfarin/Acenocoumarol and other coumarin derivatives are widely used for DVT treatment. To determine the appropriate dose of oral anticoagulants is a challenge due to narrow therapeutic index and a wide inter-individual variability in dose requirement and patients may develop thromboembolism or bleeding associated with under-dosing or over-dosing respectively.(3)

Warfarin is a “racemic mixture of R- warfarin and S-warfarin”. S-warfarin is three to five times more potent in its pharmacodynamics effect than R-warfarin. CYP2C9 enzyme metabolizes S-Warfarin and CYP-450 enzyme metabolizes R-Warfarin(4). By inhibiting the vitamin K epoxide

reductase complex 1 (VKORC1), Warfarin antagonizes functionally reduced vitamin K to be converted into oxidized vitamin K, hindering the active clotting factors formation from premature clotting factors, causing an anticoagulation effect.

Studies have consistently shown the role of both genetic and non-genetic factors in explaining the wide inter-individual variability in warfarin/Acenocoumarin dose. Genetic variation in the VKORC1 (drug target) and CYP2C9 (drug metabolizing enzyme) have been shown to account for 30-50% variability in the drug dose (4).

Therefore studying the genotypes of CYP2C9 and VKORC1 in Deep Vein Thrombotic patient is of key importance for fixing the anti-coagulant dose to reduce the adverse side effects and for effective treatment. Since CYP2C9 is important for the clearance of the anticoagulant drugs from the system this pilot study aims to rule out the presence of defective allele frequency in South Indian population.

AIM AND OBJECTIVES

- To screen the association of CYP450 2C9 genotypes to Acenocoumarol sensitivity in South Indian DVT cases.

REVIEW OF LITERATURE

Deep vein thrombosis is the presence of thrombus with accompanying inflammation in a vein. Venous thrombi are usually formed at the site of stasis. Role of platelet in the formation of venous thrombi is minimal. Venous thrombi usually occur in superficial or deep venous system of lower limb. Thrombi occurs in Superficial veins can cause congestion, swelling and pain. Deep vein thrombus is more prone for embolization.

Fate of thrombus

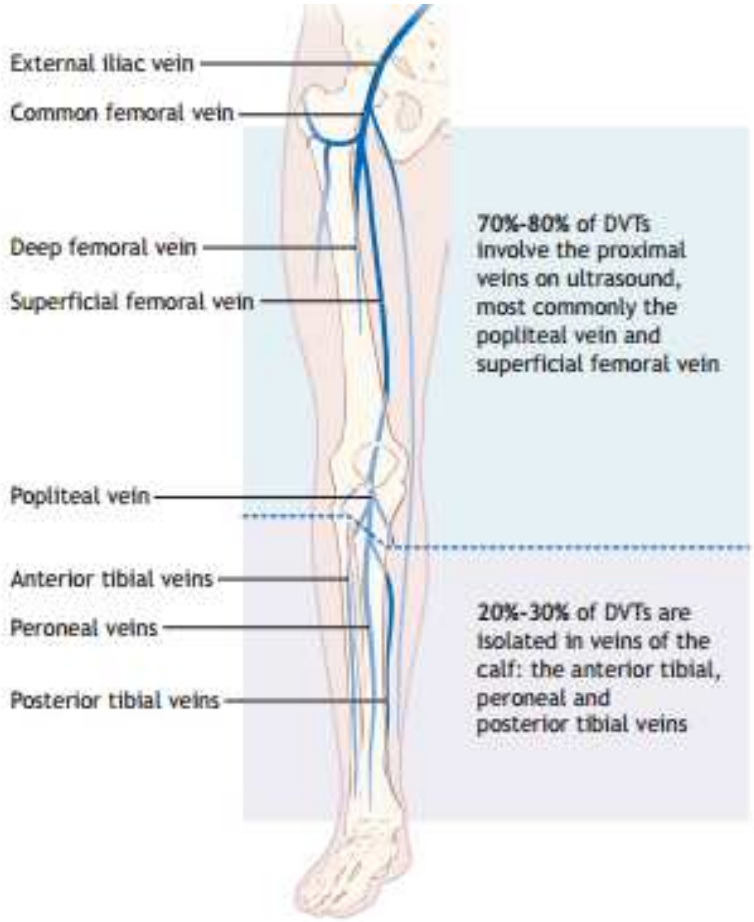
Thrombi may propagate, resolve or embolize

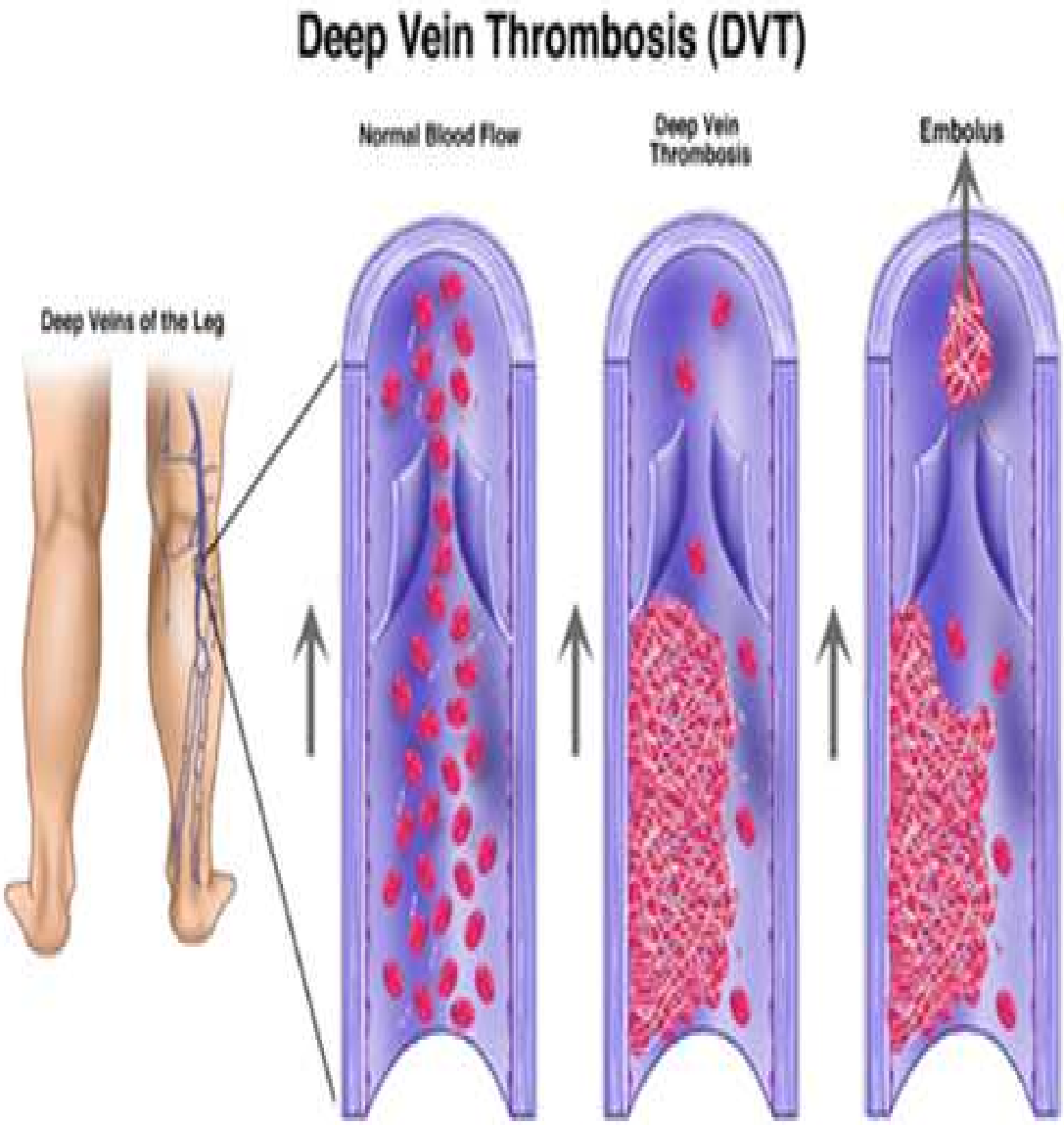
- Propagation of thrombus leads to vessel obstruction
- Dislodge of fragment of thrombi distal to circulation is called embolization
- Thrombi may be resolved due to endogenous fibrinolytic system

Deep vein thrombosis (DVT) is the main cause of life threatening pulmonary embolism (PE). Most thrombi form in the deep calf veins – in the valve sinuses of the soleal veins or behind the valve cusps in the posterior and anterior tibial vein (the muscles of the calf and the thigh). It leads to pain and swelling in the leg and may cause serious complications like PE.

DVT and pulmonary embolism together are known as venous thromboembolism (VTE). DVT in popliteal vein, femoral vein and iliac branches contributes to 20% of DVT incidences and 95% of pulmonary embolism. Annual incidence is 1/ 1,000 worldwide is affected by DVT. So it is important to prevent proximal DVT that leads to fatal PE as a complication.

In cases like Paget-Schrötter disease, venous thrombosis occurs in the upperlimb. “DVT and PE are two aspects of the disease known as venous thromboembolism”.




Deep Vein Thrombosis (DVT)
Normal Blood Flow
Deep Vein Thrombosis
Embolus
Deep Veins of the Leg

Epidemiology

- Incidence
- 1/1000 for venous thrombo embolic disease (VTE)
- Estimated DVT patients are 5 million annually
- 20% have cancer as etiology
- 5,00,000 cases of pulmonary embolism (PE) develop from these DVTs
- Sex Male>Female

  Male<Female (during childbearing years)
- Ethnicity

  Asians and pacific islanders are more affected than

  Hispanics (2 to 4 times lower risk than Caucasians and African Americans)

PATHOPHYSIOLOGY

- Virchow first described the factors that predispose to DVT in 1856

  Virchow traid-The three important factors that are essential for thrombus formation are

  i) Stasis or turbulent blood flow,

  ii) Endothelial damage

  iii) Hypercoagulability

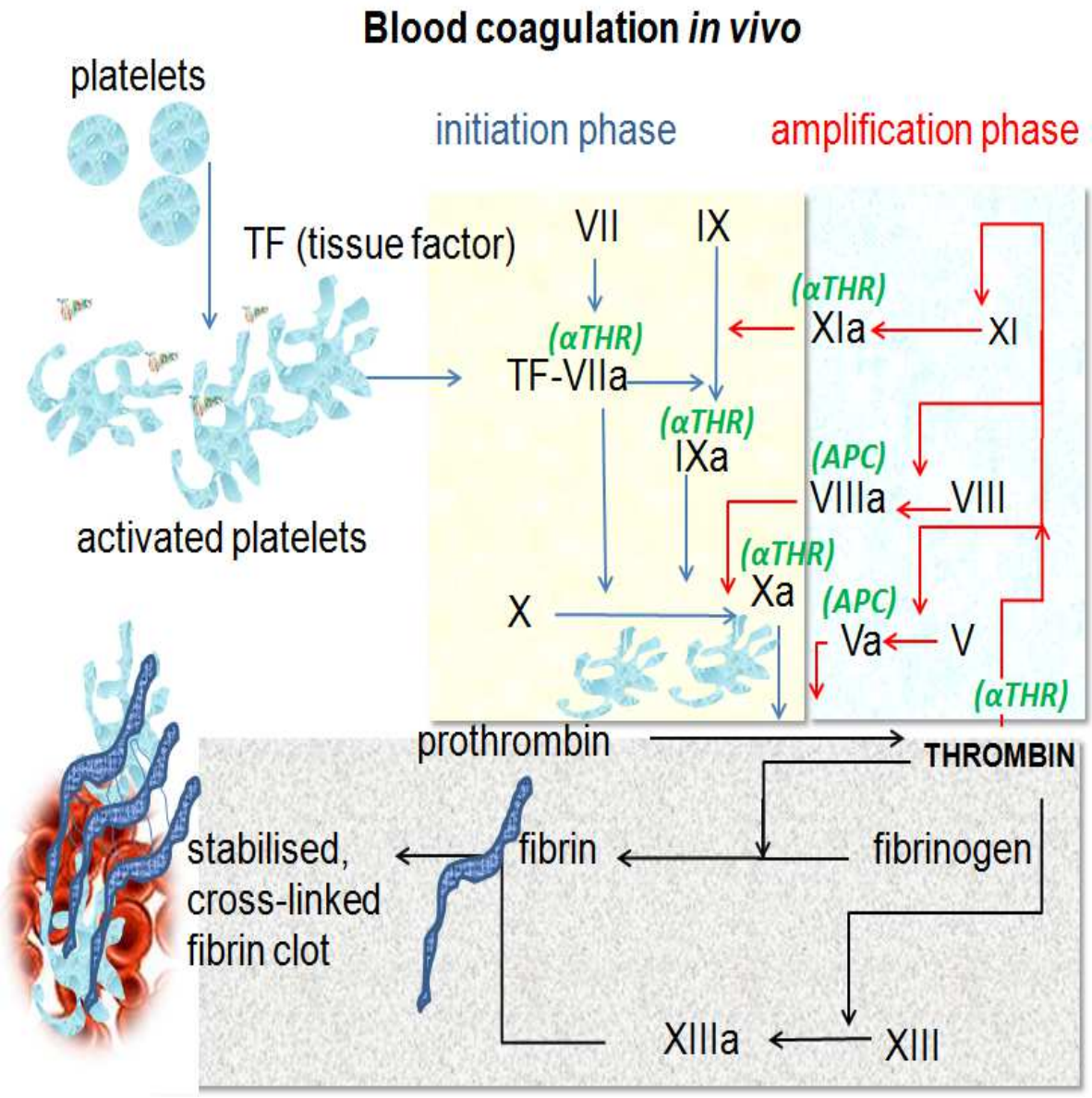
- complex interaction of congenital (inherited) factors and acquired risk factors determines individual risk

Pelvic veins and IVC can be affected by extension of lower extremity DVT. Occasionally upper limb veins are involved when indwelling central venous catheter is placed for long duration and with the rare Paget schrotter disease.

The mechanism of arterial thrombosis causing acute coronary syndrome has been studied extensively than the mechanism of development of venous thrombosis. Formation of clots in the venous system occurs without any vessel wall damage, whereas vessel wall damage initiates coagulation in arteries. Tissue factor initiates venous thrombi formation which leads to conversion of prothrombin to thrombin, followed by fibrin deposition. Venous thrombi mainly composed of RBCs and fibrin and the fibrin attaches to endothelium of blood vessel. Other components are WBCs and platelets. Even though platelets are not significantly present as in arterial thrombus, they may play a role. VTE is associated with inflammation, and 'WBCs play a role in the formation and resolution of venous clots'.

Often, Deep vein thrombosis forms in the venous valves. Hypoxemia in a valve sinus occurs due to blood flow pattern in the valves. Stasis in the venous system worsens hypoxemia, which activates pathways including HIF-1 and early growth response protein 1. Low oxygen concentration leads to

reactive oxygen species production and activates NF-B which regulates HIF-1 transcription. HIF-1 and early-growth-response protein 1 contribute to monocyte association with P-Selectin (endothelial proteins), prompting monocytes to release tissue factor-filled microvesicles, which binds to the blood vessel wall and initiate clotting.



CAUSES FOR DVT

Genetic and behavioral factors play a role in DVT. Deep vein thrombosis commonly occurs in elderly people above sixth decade, even though it occurs in all age groups. Having a family history of DVT in parents

or siblings can increase DVT risk. Smoking and obesity are important risk factors in DVT formation.

Venous thrombosis may develop when blood flow is slow or stasis occurs. Risk factors include (5, 6, 7, 8,9,10):

- A pacemaker catheter that has been manipulated through the femoral vein.
- Bed ridden state
- F/H of venous thrombosis
- Pelvic fractures
- 6 months following delivery
- Obesity
- Major surgeries involving pelvis, hip, knee.

Other risk factors are

- Malignancy
- Autoimmune disorders, such as SLE
- Smoking
- Taking estrogens or contraceptive pills (higher risk with smoking)

- Long travels

The chances are more when there is one or more of the risk factors listed above.

Inactivity- In case of inactivity for long time as in case of prolonged hospitalization, bed ridden state, or long travel, blood pools in the lower extremities increases the risk of clot Injury or surgery involving lower extremities

Genetics - In most cases the risk is only there if it is associated with at least one other risk factor.

Pregnancy – risk increases in females with some inherited blood disorders.

Cancer- risk is increased with cancers of pancreas, ovary, lung, urinary tract, breast, brain, stomach.

Ulcerative colitis and other inflammatory bowel diseases

Cardiac failure

HRT in postmenopausal females

Oral contraceptive pills

Past history of deep vein thrombosis

Ob**esity**

Smoking

Height – tall stature in males

Central venous catheters

Orthopedic casts

SYMPTOMS

Signs of DVT include

pain,

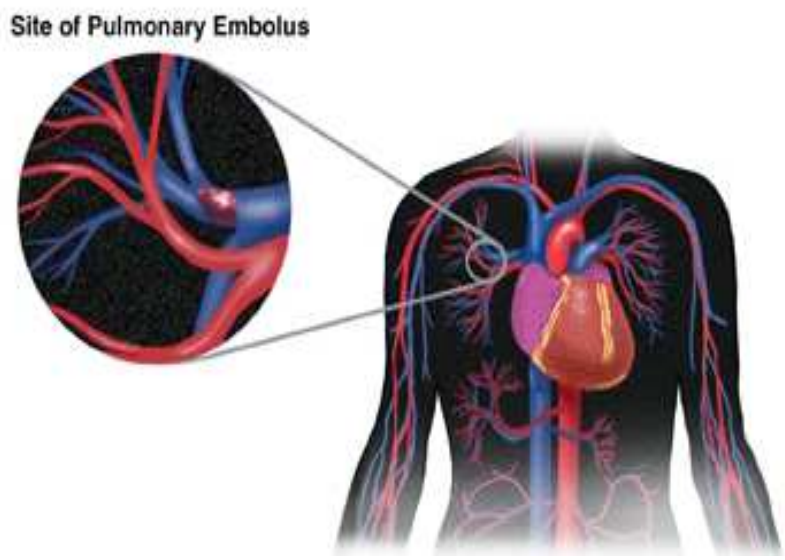
tenderness,

swelling and

Discoloration in the affected leg.

DVT symptoms can be progressive. Typically symptoms will begin in one leg. Over the course of a few days, swelling and discoloration occur in that leg. Sometimes, the condition can go undetected because deep-vein blood clots in legs can be too small to trigger medical attention.

Sometimes, patient presents with signs and symptoms of PE



- Acute onset breathlessness
- Chest pain, at rest and on exertion
- Hemoptysis
- Wheezing
- Syncope
- Unexplained anxiety
- Tachycardia

DIAGNOSIS

One or more special tests needed to rule out other problems or to confirm a diagnosis.

The two tests that are often done first to diagnose a DVT are:

- D-dimer blood test
- Doppler ultrasound exam of the legs

Criteria for diagnosis

DVT – use of Wells clinical prediction rule in establishing

Pre- test probability

CLINICAL DECISION RULE DEVELOPED BY WELLS ET AL

CLINICAL FINDING	SCORE
1 Active cancer (treatment ongoing, within	1
Previous 6 months, palliative)	
Paralysis, Paresis,	
recent immobilization of the lower extremities	1
Localised tenderness along the distribution of the	
Deep venous system	1
Entire leg swelling	1

Calf swelling by >3 cm when compared with the asymptomatic leg	1
Pitting edema	1
Collateral superficial veins	1
Alternative diagnosis as likely or greater than that of PDTV	-2

A score is obtained by summing all items that are judged to be present;

score of ≤0 = low probability of PDVT

score of 1 or 2 = moderate probability of PDVT

score of ≥3 = high probability of PDVT

Other clinical prediction rules include charlotte and Genewa / modified Genewa

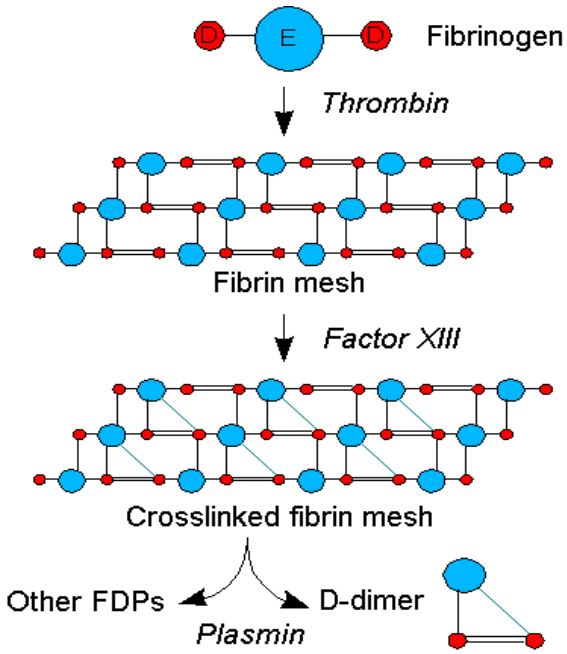
Other blood tests are following:

- Complete blood count (CBC)
- Activated protein C resistance (checks for the Factor V Leiden mutation)
- Antithrombin III levels
- Antiphospholipid antibodies
- Protein C and protein S levels

- Genetic testing to look for mutations that make patients more likely to develop blood clots, such as the prothrombin G20210A mutation

D-Dimer Test: D-Dimer presents in the circulation in small quantities normally. During the process of clotting it is produced as a breakdown product .Hence, it's presence suggest the increased clotting activity in the body.

Principle of test:



Human blood plasma usually does not have D-dimers. Activation of coagulation system produces them in blood. The D-dimer assay depends on the binding of a monoclonal antibody to a particular epitope on the D-dimer fragment. The commercially available kits rely on a different monoclonal antibody against D-dimer. The binding of the antibody is measured quantitatively quantitative measurement of antibody binding is done by one of various laboratory methods.

Detection of evidence of thrombus within the circulation: D-dimer

A D- dimer measured by enzyme-linked immunosorbent assay (ELISA) below a cut-off of 300 to 540 ng/ml (the values differ slightly from one study to another) make the diagnosis of DVT (or PE) unlikely. However, a

concentration of D-dimer above the cut-off level is not useful for making a positive diagnosis because of the large number of false positive tests.

Conventional ELISA assays are cumbersome and not suited for emergency use, which limited the practical utility of D-dimer measurements until the development of rapid ELISA assays. These provide the best balance of sensitivity and specificity among the various assays for the safe diagnostic handling of patients with suspected DVT and PE.

Activated protein C resistance (APCR)

The disorder can be

Acquired or inherited

Autosomal dominant inheritance pattern

64% of patients with VTE might have activated protein C resistance.

Antithrombin III is a blood test that measures the amount of antithrombin III (AT III), and risk of thrombosis occurs when there is deficiency of AT III in blood, or it is less active.

Lupus anticoagulants are antibodies against substances in the lining of cells. These substances prevent blood clotting in a test tube. They are called phospholipids. Persons with these antibodies may have an abnormally high risk of blood clotting.

Duplex ultrasound: Painless and non-invasive, ultrasound tests require no radiation but require a skilled person to obtain accurate results. The drawback of this test is less sensitive in finding pelvic thrombosis which are deeply situated.

Venography: Detection of the physical presence of thrombus in leg veins. The ‘gold standard’ is contrast venography, but this can be unpleasant for patients, time consuming for radiology departments, and expensive. This has driven the search for acceptable noninvasive methods of diagnosis and contrast venography is now rarely performed, except as part of research protocols. In most centres contrast venography has been replaced by B-mode ultrasonography as the preferred first line diagnostic technique.

Magnetic resonance imaging (MRI): This is useful in finding out deeply situated pelvic thrombus.

COMPLICATIONS

Other complications by PE

- ✓ Palpitations
- ✓ Cardiac failure or cardiogenic shock
- ✓ Breathing difficulties
- ✓ Pulmonary hypertension.

Other rare DVT Complications include:

Renal vein thrombosis

- ✓ Heart attack
- ✓ Stroke
- ✓ It is important to treat pulmonary embolism immediately.

TREATMENT

Surgical intervention for DVT is needed in rare cases like large clot obstructing a major blood vessel, causing severe symptoms. But, Surgery itself increases the risk of new thrombus formation.

Goals of Treatment for DVT to prevent

- ✓ Extension of thrombus.
- ✓ embolism formation
- ✓ complications like post thrombotic syndrome such as leg pain and swelling
- ✓ recurrences

Medical management for DVT

DVT and PE are aspects of the same disease-VTE. Forty percent of patients with DVT without clinical evidence of PE have evidence of emboli on lung scanning. The principles of therapeutic coagulation are the same for both. In proximal DVT and PE this has involved immediate anticoagulation with heparin followed by a period of warfarin. Distal DVT can be managed in the same way, but an alternative strategy is to use serial noninvasive testing which only reliably detects proximal thrombosis, to ensure that suspected distal thrombosis does not extend above the knees, withholding treatment if it does not.

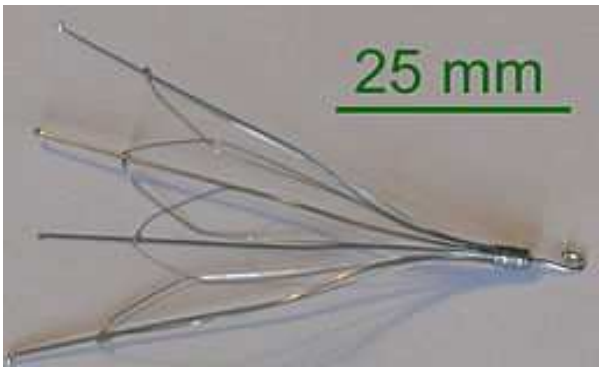
There is clear evidence that immediately acting anticoagulation is needed in the initial phase and that anticoagulation with oral vitamin K antagonists alone is inadequate. Warfarin can be commenced on the first day and heparin is continued for 5 days or until the INR is greater than 2.0 for 2

consecutive days, whichever is the longer. Extending the period of heparinization from 5-10 days is not more effective and increases the risk of HIT. However, massive PE or severe iliofemoral thrombosis a long period of heparin therapy may be considered(11). A 3 to 6-month course of warfarin oral tablets is also prescribed to prevent a recurrence of DVT. For those with recurrent DVTs, anticoagulant medication is usually taken for lifelong.

Surgical intervention for DVT is needed in rare cases like large clot obstructing a major blood vessel, causing severe symptoms. But, Surgery itself increases the risk of new thrombus formation. In open surgical thrombectomy, complete removal of thrombus from the affected veins is done. This procedure needs multiple incisions and procedural maneuvers, like balloon embolectomy, irrigation and creation of arteriovenous fistula, and still this may end in failure. Patients are at risk of wound complications and later they may need arteriovenous fistula ligation. But underlying etiology is not corrected. So, it has limited role in the acute DVT management. For this reason, surgical intervention is resumed for patients with extensive DVT and Phlegmasia(11,12).

Thrombolysis - TPA (tissue plasminogen activator) is used for this procedure. Due to serious side effects like bleeding, they are used only the life is at risk.

Inferior vena cava filter -Tiny umbrella-like device is used to filter venous thrombus. So that venous flow is maintained normally.



Inferior vena cava filter

Catheter directed thrombolysis (clot busting) treatment:

It is done by interventional radiologists under imaging guidance. It is designed for immediate lysis of clot, restoring blood flow immediately, preserving valve function and minimizing complications. Thrombolysis is done after placing the tip into the clot. Venography is done to look for narrowing of the vein and balloon angioplasty or stent placement is done with interventional radiologist.

Compression stocking – which helps to reduce pain and swelling. It also prevents the development of long term DVT complications like post-thrombotic syndrome.

Post thrombotic syndrome

Irreversible damage in the affected vein and valves

Stasis of blood

Chronic leg pain

Fatiguability

Swelling severe skin ulcers

DRUGS USED TO TREAT DVT

HEPARIN (UH)

It is a highly sulfated glycosaminoglycan, which is as an injectable in the treatment of DVT and PE. It is also used in the treatment of ischemic heart disease, cerebrovascular accidents like stroke and CVT.

Mast cells store heparin as secretary granules and tissue injury releases heparin into the circulation as a defense mechanism. Heparin sulphate proteoglycans derived from endothelial cells play a role in anticoagulation.

MECHANISM OF ACTION

Heparin administration

⇩

Heparin binds to Antithrombin III

Reactive site loop flexibility increases

AT becomes 1000 fold more active

Inhibition of the activated coagulation factor

which include Thrombin, factor Xa, factor IXa, IXa, XIIa,XIIIa

HEPARIN METABOLISM (13)

Saturable mechanism	Unsaturable mechanism
Through RE system and endothelial cells	Excreted through kidney
To which heparin binds with high affinity	-
Acts in low dose	Acts in higher dose

WARFARIN

Campell and Link (1939) identified bishydroxycoumarin (Dicoumarol). A synthetic congener, a potent rodenticide was developed in 1948.

The oral vitamin K antagonists have been the mainstay of long term anticoagulant therapy. Warfarin is the commonest antagonist given. Acenocoumarol having shorter half-life and Phenindione also are used. Warfarin takes a number of days to become effective, during which period heparin is given. When warfarin is started, the vitamin K dependent factors fall according to their half-lives. Factor VII and protein C have the shortest half-lives, so that despite a prolongation of the INR due to factor VII deficiency, warfarin may initially be procoagulant. This is the mechanism for the rare problem of warfarin induced skin necrosis most often described on those with protein c deficiency(14).

Chemistry

Warfarin is the prototype of anticoagulant**.** Numerous anticoagulants have been synthesized as 4-hydroxycoumarin derivatives and of the related compound indan-1’3-dione.Only coumarin derivatives are used widely. The 4-hydroxy coumarin residue, with a nonpolar carbon substituent at the position 3, is the minimal structural requirement for activity. This carbon is asymmetrical in warfarin and in phenprocoumarin and acenocoumarol.

The R-and S-enantiomers differ in anticoagulant potency, metabolism, elimination, and interactions with other drugs. Commercial preparations of

the anticoagulants are racemic mixtures. No advantage of administering a single enantiomer has been established.

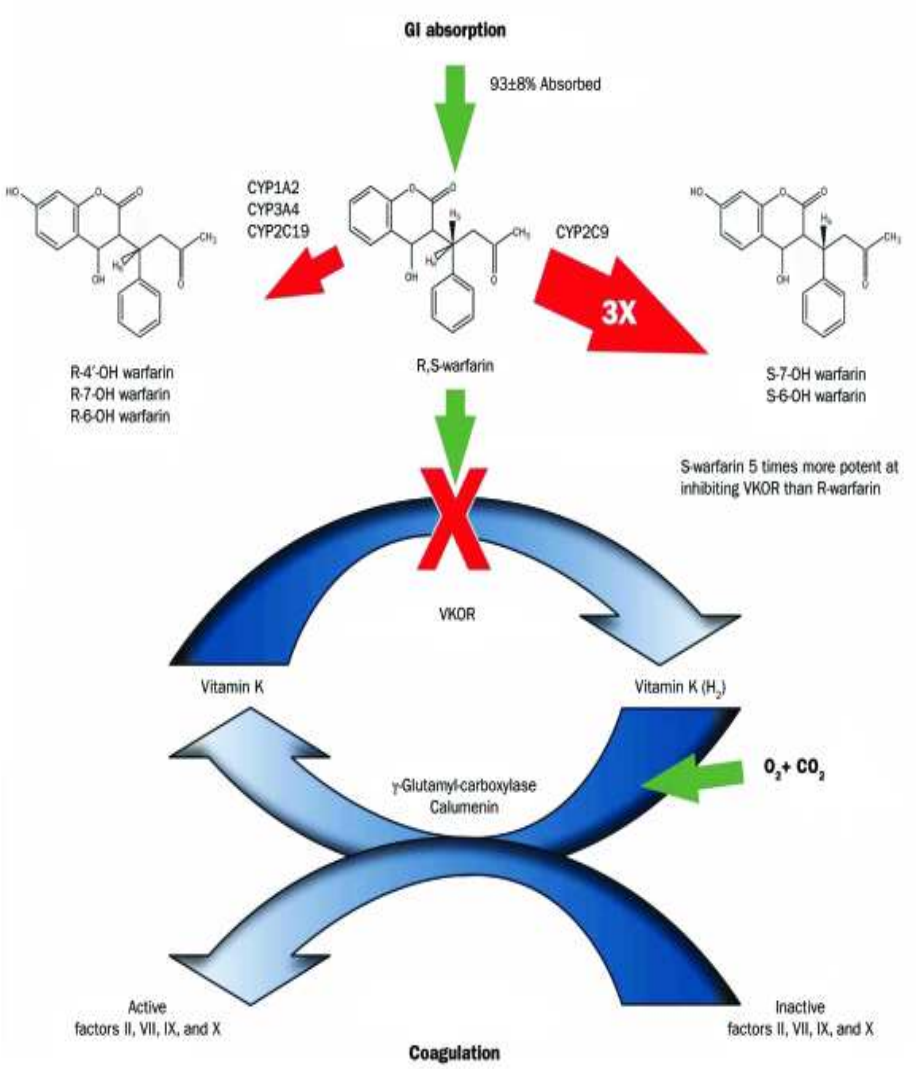
Mechanism of action

The oral anticoagulants act indirectly by interfering vitamin K dependent clotting factors synthesised in liver. They block the active hydroquinone form of vitamin K regeneration, the cofactor required for gamma-carboxylation of glutamate residues of prothrombin and factor VII, IX, X. Carboxylated factors then bind to Ca 2+ and coagulation sequence proceeds. During gamma carboxylation, active vitamin K is oxidized to an epoxide, which must be reduced back to vitamin K hydroquinone to become active once again by the enzyme vitamin K reductase. This enzyme is inhibited by oral anticoagulants. S- warfarin is metabolized by CYP2C9. Common genetic polymorphism in this enzyme can influence warfarin metabolism. Polymorphisms in the C1 subunit of vitamin K reductase (VKORC1) also can affect the susceptibility of the enzyme to warfarin induced inhibition, thereby influencing warfarin dosage requirements(15, 16).

Congenital defeciencies of the procoagulant proteins to these levels cause mild bleeding disorders. It takes some days to attain full antithrombotic effect of Warfarin, as the t1/2 of factor II is longer. But PT will be prolonged immediately after administration because of the shorter half-life of factor VII that is rapidly reduced.

The approximate t1/2 in hours follows

Coagulation factors	Half-life (hr)
Factor VII	6
Factor IX	24
Factor X	36
Factor II	50
Protein C	8
Protein S	30



Dosage

The usual adult dosage of warfarin is 2-5mg for 2-4 days followed by 1-10 mg as indicated by measurements of the International Normalised Ratio (INR) a value derived from the patient's PT. As indicated later common genetic polymorphisms render patients more or less sensitive to warfarin. A lower initial dose should be given to the patients with increased risk of bleeding, including the elderly. Warfarin is administered orally. Age correlates with increased sensitivity to the drug. Warfarin can also be given

intravenously without dose modification. Intramuscular injection is not recommended because of the risk of hematoma formation.

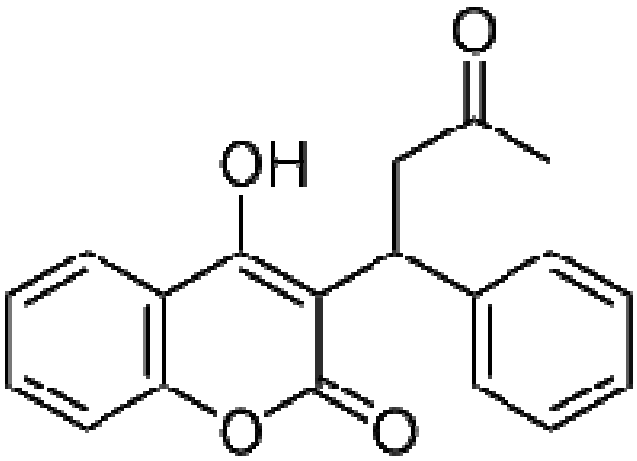
Absorption

The bioavailability of warfarin is nearly complete when the drug is administered orally, intravenously or rectally. Repeated skin contact with the solution of warfarin used as a rodenticide may cause bleeding. Food can decrease the rate and extent of absorption. Warfarin usually is detectable within one hour of its oral administration and concentration peak in 2-8 hours.

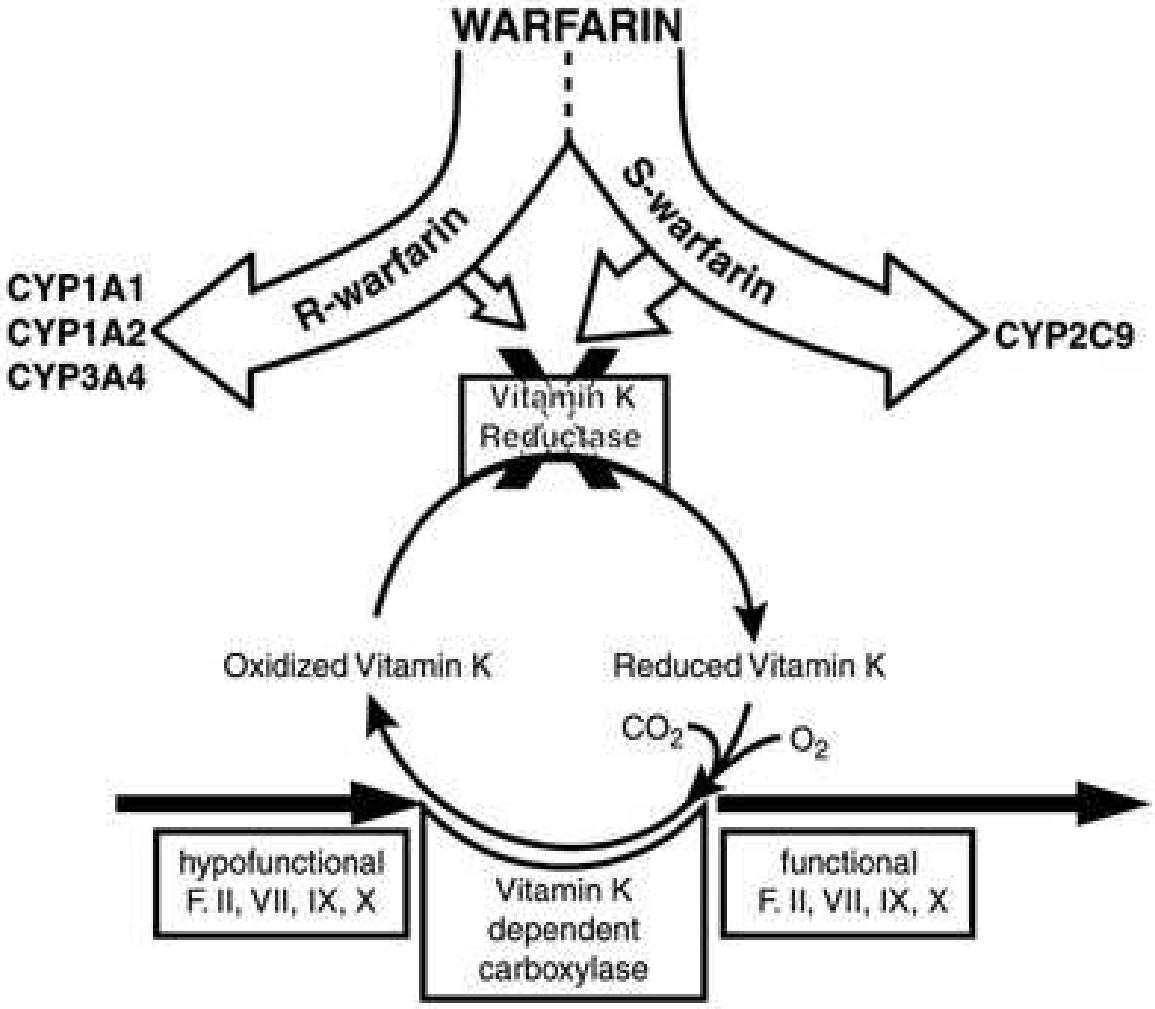
Distribution

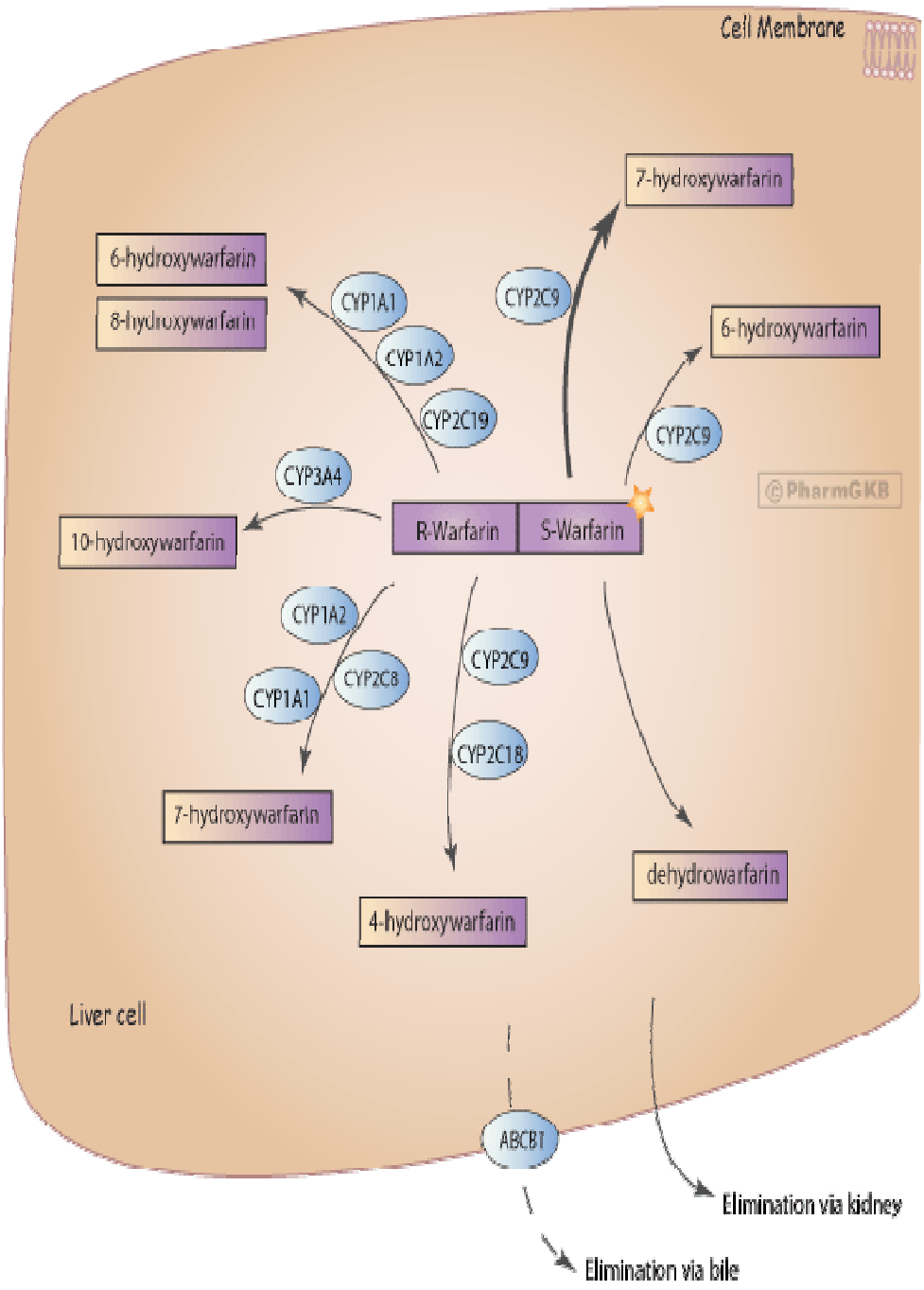
Warfarin is binding almost completely to plasma proteins, principally albumin, and the drug is rapidly distributed into the albumin space. Foetal plasma concentrations approach the maternal values, but active warfarin is not found in milk (unlike other coumarins and idandiones). Therefore, warfarin can safely be administered to nursing mothers.

Warfarin Metabolism



Warfarin is administered as a racemic mixture of R-S warfarin. S-warfarin is 3-5 folds more potent than R- warfarin and is metabolized principally by CYP2C9.


WARFARIN
R-warfarin
S-warfarin
CYP1A1
CYP1A2
CYP3A4
CYP2C9
Vitamin K
Reductase
Oxidized Vitamin K
Reduced Vitamin K
CO_2
O_2
hypofunctional
F. II, VII, IX, X
Vitamin K
dependent
carboxylase
functional
F. II, VII, IX, X



Individuals with the CYP2C9*2 and CYP2C9*3 polymorphisms have decreased clearance of S-warfarin and therefore also an increased plasma concentration ratio when compared to R-warfarin. In patients with allelic variants the relative concentration of S-warfarin increases in patients homozygous for the CYP2C9*3 polymorphisms(14,15).

CYP2C9 and VKORC1 single nucleotide polymorphisms causes variable response to warfarin. The enzyme encoded by CYP2C9 is associated with reduced activity and decreased requirement of drug dose

Inactive warfarin metabolites are excreted in urine and stool. The average rate of clearance from plasma is 0.045 ml/mt/kg. The t1/2 varies (25-60 hours) with a mean of 2-5 days.

Drug and other interactions

Factors that cause a decreased effect of oral anticoagulants include:

- Reduced drug absorption by cholestyramine which binds with the drug in the GI tract
- Increased distribution volume and a short t1/2 secondary to hypoproteinemia as in nephrotic syndrome
- Enzyme inducers like barbiturates, carbamazepine or rifampin
- vitamin K rich foods or supplements
- Pregnancy

The PT can be shortened in any of the above cases.

Many drugs interact with warfarin. Such that patient education and constant vigilance are essential. Close monitoring of the INR is advised when concomitant medication is altered.

Drugs that potentiate warfarin

Amiodarone

Omeperazole

Cimetidine

Clofibrate

Erythromycin

Cotrimoxazol

Flucanazole

Isoniacid

Metronidazole

Micanazole

Propranolol

Piroxicam

Propafenone

Statins

Sulphinpyrazone

Drugs that inhibit warfarin

Barbiturates

Carbamazepine

Chlordiazepoxide

Cholestyramine

Griseofulvin

Rifampicin

Sucralfate

Relative deficiency of vitamin k may result from inadequate diet (eg: postoperative patients on parental fluids) especially when coupled with the elimination of intestinal flora by antimicrobial agents. Vitamin k gut bacteria are an important source of this vitamin. In addition to an effect on reducing intestinal flora, cephalosporin containing heterocyclic side chains also inhibits steps in the vitamin cycle. Low concentrations of coagulation factors may result from impaired hepatic function, congestive heart failure or hyper metabolic states such as hyperthyroidism. Elderly patients are more sensitive to oral anticoagulants.

Resistance to warfarin

- (Some patients require>20 mg/d of warfarin to achieve therapeutic INR).
- Excessive vitamin K intake from the diet or parental supplementation.
- Noncompliance
- Laboratory error
- Hereditary warfarin resistance
- Mutations in the VKORC1 gene

Sensitivity to warfarin

Approximately 10% patients require <1.5 mg/d of warfarin to achieve an INR target of 2-3. As indicated earlier these patients often possess variant alleles of CYP2C9 or variant VKORC1 haplotype, which affect the pharmacokinetics or pharmacodynamics of warfarin respectively (daly and King,2003).

Adverse effects

Bleeding

Incidence is <3% per year in patients treated with a target INR of 2-3. Risk factors for bleeding are increasing age, a history of stroke, a history of gastro intestinal bleed, anemia, renal impairment, diabetes, and recent MI. A major problem is in the starting and stopping of other medication. The ICH risk increases dramatically with an INR >4, especially in elderly people. In a large outpatient anticoagulation clinic, the most common factors associated with a transient elevation of the INR to a value of >6 were uses of a new medication known to potentiate warfarin (eg;acetaminophen)(17), advanced malignancy, recent diarrhoeal illness, and taking more warfarin than prescribed. Patients must be informed of the signs and symptoms of bleeding and laboratory monitoring should be done at frequent intervals during intercurrent illnesses or any change or medication or diet.

If the INR is above the therapeutic range but <5 and the patient is not bleeding or in need of a surgical procedure, warfarin can be discontinued temporarily and restarted at a lower dose once the INR is within the therapeutic range (15). If the INR is more or equal to 5, vitamin K (phytanadione) can be given orally at a dose of 1-2.5mg (for 5-9) or 3-5mg (for INR>9). Then doses of oral vitamin K generally cause the INR to fall substantially within 24-48 hours without rendering the patient resistant to further warfarin therapy. Higher doses or parenteral administration may be required if more rapid correction of the INR is necessary. The effect of vitamin K is delayed for at least several hours because reversal of anticoagulation requires synthesis of fully carboxylated coagulation factors. If immediate hemostatic competence is necessary because of serious bleeding or profund warfarin overdosage (INR>20), adequate concentration of vitamin K dependent coagulation factors can be restored by transcription of fresh frozen plasma (10-20ml/kg) supplemented with 10 mg of vitamin K, given by intravenous infusion. Transfusion of plasma may need to be reported because of transfused factors (particularly Factor VII) are cleared from the circulation more rapidly than the residual oral anticoagulant. Vitamin K administered intravenously carries the risk of anaphylactoid reaction and therefore should be used cautiously and administered slowly. Patients who receive high doses of vitamin K may become unresponsive to

warfarin for several days, but heparin can be used if continued anticoagulation is required.

Birth defects

Abortions

Nasal hypoplasia and stippled epiphyseal calcifications

CNS abnormalities

Foetal or neonatal haemorrhage

Intrauterine death

Vitamin K antagonists should not be used during pregnancy, but, Heparin, LMWH, or fondaparinux can be used safely in this circumstance.

Skin necrosis

Warfarin induced skin necrosis is a rare complication charecterized by the appearance of skin lesions 3-10 days after treatment is initiated. The lesions typically on the extremites, but adipose tissue, the penis and the female breast also may be involved. Lesions are charecterized by widespread thrombosis of the microvasculature and can spread rapidly in response to the initial dose of vitamin k antagonists. It has been proposed that the dermal necrosis is a manifestation of a temporal imbalance between the anticoagulant protein C and one or more of the procoagulant factors and is exaggerated in patients who are partially deficient in protein C or protein S. However, not all

patients with heterozygous deficiency of protein C or protein S develop skin necrosis when treated with warfarin, and patients with normal activities of these patients can also be affected. Morphologically similar lesions can occur in patients with vitamin k deficiency.

Other toxicities

Purple toe syndrome after 3-8 weeks of therapy

Other infrequent reactions include alopecia, urticaria, dermatitis, fever, nausea, diarrhoea, abdominal cramps, and anorexia.

"Warfarin can precipitate the syndromes of venous limb gangrene and multicentric skin necrosis when given to patients with heparin induced thrombocytopenia who are not receiving a parenteral anticoagulant" (Warkentin 2003).

Clinical use

Vitamin K antagonists are used to prevent the progression or recurrence of acute DVT or pulmonary embolism following an initial course of heparin. As per Geerts et al., 2008, "they also are effective in preventing venous thromboembolism in patients undergoing orthopaedic or gynaecological surgery, recurrent coronary ischemia in patients with acute myocardial infarction, and systemic embolization in patients with prosthetic heart valves or chronic atrial fibrillation. Specific recommendations for oral

anticoagulant use for these indications have been reviewed". "A higher target INR (eg; 2.5-3.5) generally is recommended for patients with high risk mechanical valve" (Hirsh et al 2003)(18).

For treatment of acute thromboembolism, Heparin, LMWH, or fondaparinux usually is continued for atleast 5 days after warfarin therapy is begun and until the INR is in the therapeutic range on two consecutive days. This overlap allows for adequate depletion of the vitamin K dependent coagulation factors with long half -life especially factor II. Frequent INR measurements are indicated at the onset of therapy to guard against excessive anticoagulation in the unusually sensitive patient. The testing interval can be lengthened gradually to weekly and then monthly for patients on long term therapy whose test results have been stable.

Initiation of anticoagulation with warfarin

The INR equals $(PT/MNPT)^{ISI}$ where MNPT is the (mean normal) control PT and ISI is the international sensitivity index of the thromboplastin and used in the assay. For the treatment of DVT and PE, target INR should be 2.5 (range 2.0 to 3.0).

If the initial coagulation tests are not prolonged, it has been customary to start 10mg of warfarin or 2mg of Acenocoumarol the first day evening and check the INR the following morning, adjusting the dose according to the

daily INR results until the INR is stable. When INRs are stable they may go for up to 8 weeks between INR checks. If the INR is unstable patients are seen more frequently, but it should be noted that with warfarin/Acenocoumarol it takes approximately one week (5times the half-life) to reach a new study state after dose adjustment, hence more frequent dosage alteration is inadvisible.

Monitoring Anticoagulant Therapy: The INR (International Normalized Ratio)

To monitor therapy, a blood sample is obtained, and the PT is determined along with that of a sample of normal pooled plasma. Formerly, the results were reported as a simple ratio of the two PT values. However, this ratio can vary widely depending on the thromboplastin reagent and the instrument used to initiate and detect clot formation. The PT is prolonged when the functional levels of fibrinogen, factor V, or the vitamin K-dependent factors II, VII, or X are decreased. Reduced levels of factor IX or proteins C or S have no effect on the PT. PT measurements are converted to INR measurements by the following equation:

where INR = international normalized ratio

ISI = international sensitivity index

The ISI value, supplied by the manufacturer of the regent, indicates the relative sensitivity of the PT (determined from a given batch of thromboplastin) to decreases in the vitamin K-dependent coagulation factors in comparison with a World Health Organization human thromboplastin standard. Reagents with lower ISI values are more sensitive to the effects of vitamin K antagonists (i.e., the PT is prolonged to a greater extent in comparison with that obtained with a less-sensitive reagent having a higher ISI). Ideally, the ISI value of each batch of thromboplastin should be confirmed in each clinical laboratory using a set of reference plasmas to control for local variables of sample handling and instrumentation.

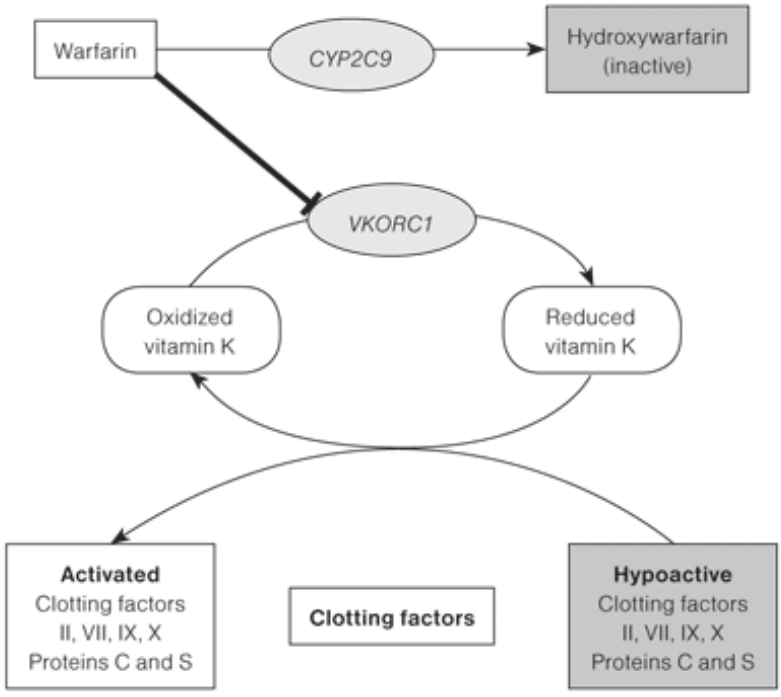
As per Moll and Ortel, 1997, “The INR does not provide a reliable indication of the degree of anticoagulation in patients with the lupus anticoagulant, in whom the PT and other phospholipid-dependent factors are prolonged at baseline. In these patients, a chromogenic factor X assay or the prothrombin-proconvertin time assay may be used to monitor therapy”

PHARMACOGENETICS OF WARFARIN

Souto JC et al., 2000 says, “Single nucleotide polymorphisms in the cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKOR)

genes have been shown to have a significant effect on warfarin dose requirement”(19).

“Warfarin is administered as a racemic mixture of (S) and (R) warfarin”. As per NA Limdi *et al*., 2008 “S-warfarin is metabolized by CYP2C9 and CYP2C8, CYP2C18, CYP2C19 serving as minor pathways. R-warfarin is mainly metabolized by CYP1A2 and CYP3A4 with CYP1A1, CYP2C8, CYP2C18, CYP2C19 and CYP3A4/5 serving as minor pathways”(20)



CYTOCHROME P450 GENES

Cytochrome P450 genes produce enzymes that are involved in the synthesis and metabolism of various molecules and chemicals within cells metabolize external substances, such as drugs that are ingested and internal substances, such as toxins that are formed within cells. There are approximately 60 CYP genes in humans.

Larsen TB *et al*., 2003 says “Common variations (polymorphisms) in cytochrome P450 genes can affect the function of the enzymes. Depending on the gene and the polymorphism, drugs can be metabolized quickly or slowly. If a cytochrome P450 enzyme metabolizes a drug slowly, the drug stays active longer and less is needed to get the desired effect. A drug that is quickly metabolized is broken down sooner and a higher dose might be needed to be effective”(21). Cytochrome P450 enzymes account for 70 to 80% of enzymes involved in drug metabolism.

CYP GENE FUNCTIONS

These enzymes are divided into two major groups

- "Steroidogenic CYP enzymes”
- "Xenobiotic CYP enzymes”

Steroidogenic CYP enzymes

- Phylogenitically older
- Present even in single cell organisms
- Located in the mitochondria of cells
- Responsible for the synthesis of steroids and other substances necessary for the maintenance of cell wall integrity
- Defeciency due to genetic mutation is incompatible with life

Xenobiotic CYP enzymes

- Evolved fron steroidogenic CYP enzymes over 1 billion year ago
- Located in the smooth endoplasmic reticulum of cells
- Ability of these enzymes to metabolize foreign (ie , Xeno) biological substance

CYP2C9 Gene (cytochrome P450, family 2, subfamily C, polypeptide 9)

Location:

10q24

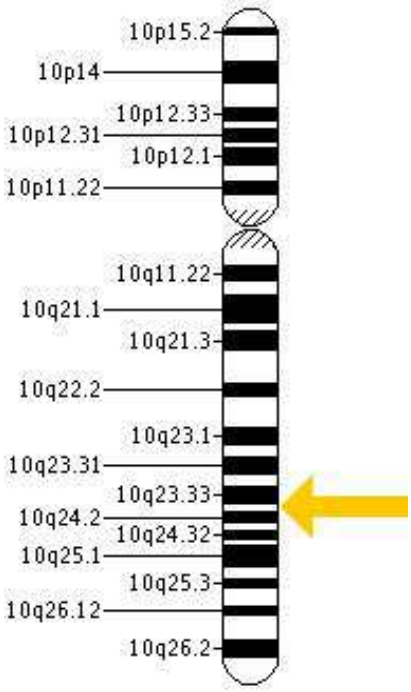
Sequence:

Chromosome: 10; NC_000010.10 (96698415..96749148)

Chromosome10-

NC_000010.10

"The CYP2C9 gene is located on the long (q) arm of chromosome 10 at position 24 from base pair 96,698,414 to base pair 96,749,147".



"The human cytochrome CYP2C9 gene spans a region of approximately 55 kilobases and is composed of nine exons" (De Morais et al., 1993). The gene resides on chromosome 10 (q24) and is clustered among other closely related 2C-genes in the order: Cen-2C18-2C19-2C9-2C8-Tel.

Three distinguishable phenotypes based on drug metabolism efficiency are estimated by genotypes.

Extensive metaboliser

Individuals with the expected normal metabolic phenotype, associated with two functional alleles or heterozygosity for a decreased functional allele.

Intermediate metabolizer

Individuals with two decreased functional alleles or with one functional alleles or with one functional allele and one non-functional allele.

Poor metabolizer

Individuals who lack active enzyme and are therefore not expected to metabolize via CYP2C9 associated with two non-functional alleles or one decreased function and one non functional allele.

Souto JC *et al.*, 2000)says, " Changes in the amino acid sequence of CYP2C9 can affect both the activity and substrate specificity of CYP2C9. Previously, three alleles were identified in the Caucasian population: *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3"(19)*.

Gene	Variant	Allele	Protein change	Effect
CYP2C9	C.430C>T	*2	R144C	Decreased activity
CYP2C9	C.1075A>C	*3	I345L	No activity

The *CYP2C9*1* allele

- encodes the wild-type protein
- Most common allele of 30 Cyp2C9 alleles

***CYP2C9*2* allele**

- Contains a C-to-T transition,
- Leading to substitution of cysteine by arginine at amino acid position 144
- Only having 70%activity

The *CYP2C9*3* allele

- Defined by an A-to-C nucleotide substitution
- Substitution of leucine by isoleucine at amino acid position 359
- Having 20% activity

Both *2, *3 variant alleles are associated with significantly reduced enzyme activity (23,24).

SNPs often lead to the formation of different alleles of a gene. Of the 30 CYP 2C9 alleles discovered, CYP 2C9*2 (or 430 C>T) and CYP 2C9*3 (1075A>C) are the two alleles that are considered strong risk factors for over anticoagulation.

Patients who are carriers of these two alleles (*2 and *3) metabolize S-warfarin more slowly *in vivo*, which results in the need for lower warfarin maintenance doses, compared with patients who carry the wild-type allele (20) .

Allele	Ethnicity	Frequencies
*2	Caucasians	10-20%
	Asians	1-3%
	Africans	0-6%
*3	allele	

- Less common
- <10% in all population
- Rare in African population

INFLUENCE OF CYP2C9 ON WARFARIN DOSE

As per Goodman and Gillman et al., “Patients with wild-type CYP2C9 genotype need approximately 6 mg/day, those with two copies of the *2 variant have an average daily dose of 4 mg/day and those with two copies of the *3 variant have an average daily dose of about 1.5 mg/day”.

CYP2C9 Genotypes	**warfarin dose**
Wild type	6mg/d
2 copies of *2 variant	4mg/d
2 copies of *3 variant	1.5mg/d

As per NA Limdi *et al*., 2008 “Among African Americans, CYP2C9 genotype has not demonstrated a consistent influence on warfarin dose. In Asian populations, CYP2C9*3 has been shown to be associated with lower dose requirements. NA Limdi *et al* provide a synthesis of data from studies that have evaluated the influence of both CYP2C9 and VKORC1 on warfarin dose”(20).

VITAMIN K EPOXIDE REDUCTASE COMPLEX, SUBUNIT 1

‘VKORC1 (vitamin K epoxide reductase complex, subunit 1) is a protein-coding gene’. Reduced vitamin K is the active form and required for the carboxylation of glutamic acid residues in some blood-clotting proteins. The product of this gene encodes the enzyme that is responsible for reducing vitamin K 2, 3-epoxide to the enzymatically activated form(21,22). Two pseudo genes have been identified on chromosome 1 and the X chromosome .

Location:

16p11.2

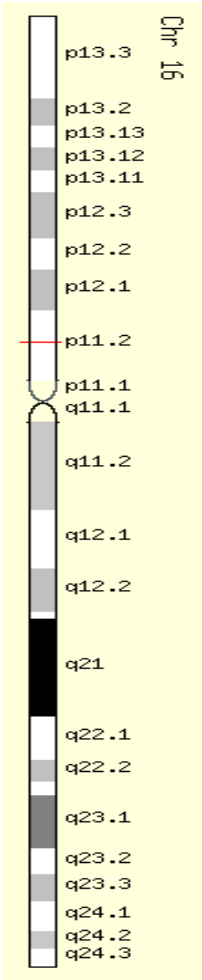
Sequence:

Chromosome: 16; NC_000016.9 (31102175..31106699, complement)

Chromosome 16 -

NC_000016.9

Warfarin works by noncompetitively inhibiting VKORC1 thus blocks the clotting cascade. Many polymorphisms in VKORC1 that influence warfarin dosing have been identified. The most commonly studied SNPs of VKORC1 include the 1173 C>T (CC is the wild-type) and 1639 G>A alleles (GG is the wild type). They are associated with a lower level of expression of VKORC1 because of their decreased translation of mRNA into proteins(25,27).



"These AA, AB, and BB haplotypes are associated with low, intermediate, and high warfarin maintenance doses, respectively".

CYP2C9 AND VKORC1 EFFECT ON WARFARIN DOSING

Lee C.R *et al*., 2000 shows, "Variability in CYP2C9 and VKORC1 alleles are associated with increased sensitivity to warfarin"(22).

As per Thomas P. Moyer *et al*., 2009, "The allelic variants found to affect warfarin sensitivity are

CYP2C9*1*1-VKORC1BB (less warfarin sensitivity than typical);

CYP2C9*1*1-VKORC1AA (considerable variance in INR throughout initiation);

CYP2C9*1*2-VKORC1AB (more sensitivity to warfarin than typical);

CYP2C9*1*3-VKORC1AB (much more sensitivity to warfarin than typical);

CYP2C9*1*2-VKORC1AB (much more sensitivity to warfarin than typical);

CYP2C9*1*3-VKORC1AA (much more sensitivity to warfarin than typical); and CYP2C9*2*2-VKORC1AB (much more sensitivity to warfarin than typical)"(26,27,28) .

GENOTYPE/ HAPLOTYPE	**CAUCASIANS**	**AFRICAN AMERICANS**	**ASIANS**	**DOSE REDUCTION COMPARED WITH WILD-TYPE (%)**
CYP2C9				
*1/*1	70	90	95	—
*1/*2	17	2	0	22
*1/*3	9	3	4	34
*2/*2	2	0	0	43
*2/*3	1	0	0	53
*3/*3	0	0	1	76
VKORC1				
Non-A/Non-A	37	82	7	—
Non-A/A	45	12	30	26
A/A	18	6	63	50

Polymorphisms in two genes, *CYP2C9* and *VKORC1* (vitamin K epoxide reductase complex, subunit 1) account for most of the genetic contribution to the variability in warfarin response. *CYP2C9* variants affect warfarin pharmacokinetics, whereas *VKORC1* variants affect warfarin pharmacodynamics. Common variations in the *CYP2C9* gene (designated *CYP2C9*2* and *3*), encode an enzyme with decreased activity, and thus are associated with higher drug concentrations and reduced warfarin dose

requirements. At least one variant allele of *CYP2C9*2* or *CYP2C9*3* is present in 25% of European-Americans, but these variants are relatively uncommon in African-American and Asian populations (Table 30–2). Heterozygosity for *CYP2C9*2* or **3* decreases the dose of warfarin required for anticoagulation by approximately 20-30% compared with "wild type" individuals (*CYP2C9*1/*1*). Homozygosity for *CYP2C9*2* or **3* can decrease the warfarin dose requirement by approximately 50-70%. Generally, the **3* allele has a greater effect than the **2* allele(35).

Range of Expected Therapeutic Warfarin Doses Based on *CYP2C9* and *VKORC1* Genotypes[†]

VKORC1	*CYP2C9*					
	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3
GG	5-7 mg	5-7 mg	3-4 mg	3-4 mg	3-4 mg	0.5-2 mg
AG	5-7 mg	3-4 mg	3-4 mg	3-4 mg	0.5-2 mg	0.5-2 mg
AA	3-4 mg	3-4 mg	0.5-2 mg	0.5-2 mg	0.5-2 mg	0.5-2 mg

[†]Ranges are derived from multiple published clinical studies. Other clinical factors (eg, age, race, body weight, sex, concomitant medications, and comorbidities) are generally accounted for along with genotype in the ranges expressed in the table.

VKORC1 -1639 G→A (rs9923231) variant is used in this table. Other co-inherited *VKORC1* variants may also be important determinants of warfarin dose.

Patients with *CYP2C9* *1/*3, *2/*2, *2/*3 and *3/*3 may require more prolonged time (>2-4 weeks) to achieve maximum INR effect for a given dosage regimen.

White people show considerable variance in CYP2C9 allele types, whereas people of Asian or African descent infrequently carry CYP2C9 allelic variants.

Warfarin Sensitivity	Genotype Combination		Prevalence	Clinical Considerations*
	VKORC1	*CYP2C9*		
Very high	A/A G/A	*1/*3, *2/*2, *2/*3, *3/*3 *3/*3	23 (2.6%)	Dose decrease and frequent INR monitoring
High	A/A G/A G/G	*1/*2 *2/*3 *3/*3	36 (4.0%)	Dose decrease and frequent INR monitoring
Moderate	A/A G/A G/G	*1/*1 *1/*2, *1/*3, *2/*2 *2/*3	238 (26.6%)	Dose decrease and frequent INR monitoring
Mild	G/G	*1/*2, *1/*3, *2/*2	109 (12.2%)	Frequent INR monitoring
Normal	G/A	*1/*1	262 (29.2%)	Likely to experience normal response to warfarin
Less than normal	G/G	*1/*1	228 (25.4%)	Dose increase may be required to maintain optimal INR
Total			896 (100%)	

METHODS AND MATERIALS

Study centre

Institute of Internal Medicine, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai

Study design

Case control study

Duration of the study

6 months

Sample

Fifty subjects with clinical evidence of Deep vein thrombosis were selected after applying inclusion & exclusion criteria from the inpatients of Internal Medicine and Vascular Surgery departments of Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai

Inclusion criteria

Patients aged between >18 of both gender with confirmed Deep Vein Thrombosis.

Exclusion criteria

Use of a fibrinolytic agent to treat the current episode of DVT and/or PE

Liver diseases and renal failure

Haematological disorders

Methodology

All cases were subjected to a detailed history taking and clinical examination based on a simple questionnaire which included complaints like pain, swelling, discoloration of lower limb, PT/INR and Doppler study of affected lower limb done.

Collection of samples

The cases recruited for this study were confirmed DVT cases based on clinical features and Doppler study. The study was explained to the patient in detail and informed consent was obtained. Detailed clinical parameters were collected from case records. Five ml of blood sample was collected from the patient in EDTA coated vacutainer tubes and transported on ice to lab.

Reagents

Tris 1M (PH 8.0)

EDTA 0.5 (PH 8.0)

TE (PH 8.0) (10 mM Tris, 1mM EDTA)

RBC lysis buffer

WBC lysis buffer

TAE 1x

Proteinase K

Phenol: chloroform:isoamyl alcohol

DNA isolation

DNA isolation was carried out by classical Proteinase K digestion and Phenol: chloroform : isoamyl alcohol method as described by Maniatis *et al.,(*1982). The blood sample was centrifuged at 3500rpm for 15min and the buffy coat which contains WBC was separated and was transferred to 2ml of centrifuge tube. To remove the residual RBCs 1ml of 1x RBC loses buffer was added and incubated for 15 min at 37˚C. The sample was centrifuged at 4000rpm for 5 min at room temperature and the supernatant was discarded. The cell pellet was washed with RBC lysis buffer for two more rounds. To the cell pellet, 500 µl of WBC lysis buffer, 5 µl of Proteinase K (1mg/ml) and 10% SDS 25 µl (Final concentration 1%) was added and mixed well. The tubes were incubated at 37˚C for overnight in water bath. After the incubation period Phenol: Chloroform: Iso-proponal (25:24:1) mix was added to the clear lysate and mixed well until it forms a milky solution. The mixture was centrifuged at 12000 rpm for 20 min at 4˚C. The upper aqueous phase which

contains the DNA was transferred to a new 2 ml centrifuge tube using wide bore pipette. One more PCI extraction and two chloroform:isoamyl alcohol extraction was done to clear the protein and residual phenol. The aquous phase was transferred to a new tube and to this 2 volume of absolute ethanol was added. The tubes were inverted gently till DNA thread appears. The DNA was collected by spooling or pelleted by spinning at 12000rpm for 10 mins at 4˚C. The supernatant was discarded and 1 ml of 70% ethanol was added to the DNA pellet to wash the residual salt and centrifuged for 12000 rpm for 10 mins at 4˚C. The supernatant was discarded and the DNA pellet was air dried. The DNA was dissolved in appropriate amount of 1X TE buffer and stored in 4˚C. The isolated DNA was quantified in UV spectrometer.

CYP2C9 Genetic polymorphism to be analyzed in this study

Gene	**Allele variants**	**SNP ID**	**Base position**
CYP2C9	*2	rs1799853	430C>T
	*3	rs1057910	1075A>C
	*4	rs1057909	1076T>C
	*5	rs28371686	1080C>G

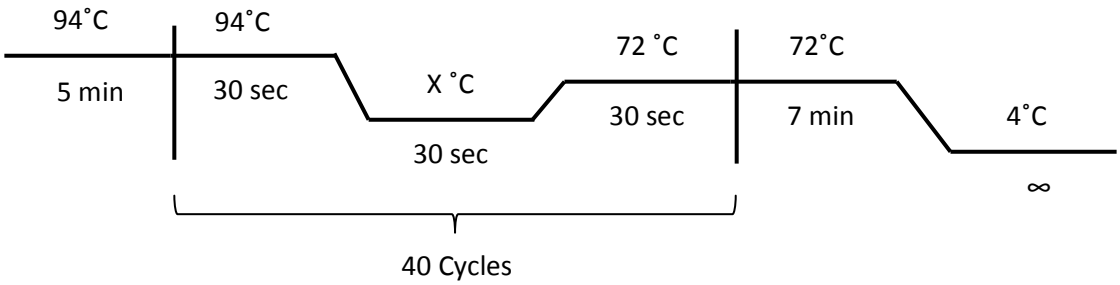
PCR amplification

CYP2C9 exon1 covering the*2 variability and exon 4 region that is having variant *3,*4,*5 were amplified using the forward and reverse primers in 20 µl reaction with 100 ng of template DNA. The reaction conditions and temperature cycling parameters were as follows:

PCR reaction mix

PCR content	**For 10µl reaction**	**Final Concentration**
10X PCR buffer	1	1X
25mM MgCl2	0.6	1.5mM
2.5µM dNTPs	0.4	100µM
2µM Forward primer	0.3	60nM
2µM Reverse primer	0.3	60nM
Taq Pol	0.05	0.02U
D. H2O	6.17	-
Template DNA	1	100ngm

Thermal cycling parameters



The PCR product was run in 2% Agarose with 0.5X TAE at 100 Volt.

Primers used to amplify CYP2C9 exon 1 region to study *2 variant

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	CATGGCTGCCCAGTGTCAGC	20	64.05	65.00
Reverse primer	AGCAAAGTTCAGGAGAACATGGGA	24	62.28	45.83

>chr10:96701876+96702348 473bp

CATGGCTGCCCAGTGTCAGCttcctctttcttgcctgggatctccctcctagtttcgtttctcttcc

tgtta

ggaattgttttcagcaatggaaagaaat

ggaaggagatccggcgtttctccctcatgacgctgcggaattttgggatg

gggaagaggagcattgaggacC*2gtgttcaagaggaagcccgctgccttgt

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tggccatatctttcattatgagtcctggttgttagctcatgtgaagcgggggtttgaagctgagagccaagggaa

tttgcacatatttgtgctgtgtg

tgtacaggcatgattgtgcgtacagtgtgggtataaaaggttcatttaaTCCCATGTT

CTCCTGAACTTTGCT

Restriction fragment length polymorphism (PCR-RFLP)

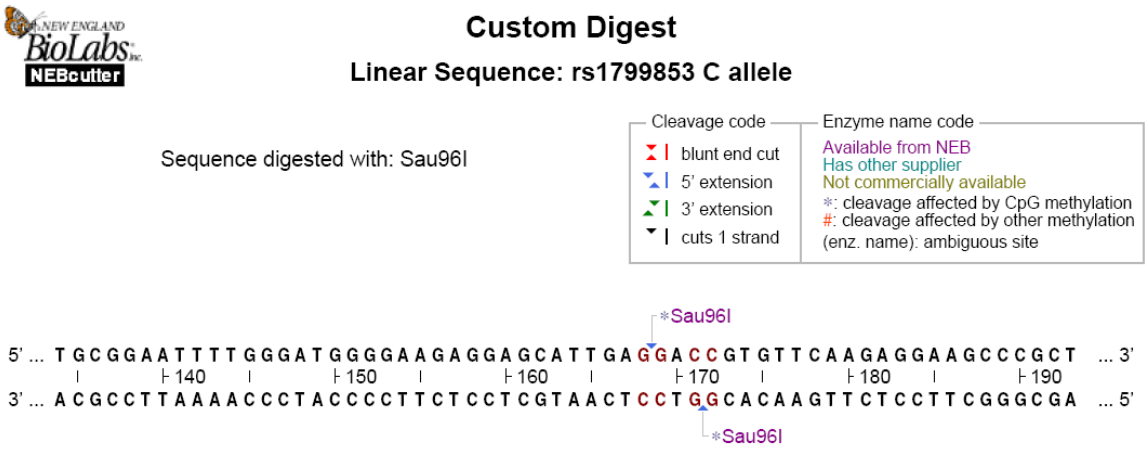
The PCR products of the CYP2C9 exon 1 was subjected to RFLP using 2Units of *SaU96I* restriction enzyme with NEB buffer 4 at 37˚C overnight to analyze the CYP2C9*2 genetic variant.

Content	**For 20ul Reaction**	**Final Concentration**
PCR product	10	-
SaU96 I Enzyme	0.4	2 Units
10X NEB buffer 4	2	1X
D. H_2O	7.6	-

Restriction digested PCR products were run in 2% Agarose gels made with 1X TAE and run at 100 V for 30 min using 1X TAE as running buffer in Mupid Ex (Takara, Japan) electrophoresis unit. Gels were stained with Ethidium Bromide and photographed using Gel documentation system (UV

Tech, USA). The genetic variants were identified based on the restriction pattern of PCR product.

Recognition sequence of SaU96I restriction enzyme is altered due to the CYP2C9 *2 variant (430C>T).



Digestion pattern of the CYP2C9*2 allele

Allele	CC	CT	TT
Digestion pattern (bp)		473	473
	305	305	
	168	168	

Primers used to amplify CYP2C9 exon 4 region to study *3, *4, *5 variants

>chr10:96740964+96741219 (256bp)

GTGTGATTGGCAGAAACCGGAGCccctgcatgcaagacaggagccacatgccctacac
aga

tgctgtggtgcacgaggtccagagatac**A**[*3]**T**[*4]tga**C**[*5]cttctccccaccagcctgccccatgcagtgacc
tgtgacattaaattcagaaactatctcattcccaaggtaagtttgtttctcctacactgcaactccatgttttcgaagt
ccccaaattcatagtatcatttttaaaCCTCTACCATCACCGGGTGAGA

To avoid interference from family members like CYP2C19) the following internal reverse primer was used for Sequencing CYP2C9 exon4 PCR product.

5`- GATACTATGAATTTGGGGACTTCG -3`

SEQUENCING

The PCR products were sequenced through a commercial service provider Macrogen Inc. South Korea using the regular forward primers and an internal reverse primer specific to CYP2C9. The sequencing chromogram was read at BioEdit V 7.0.9.0 Software.

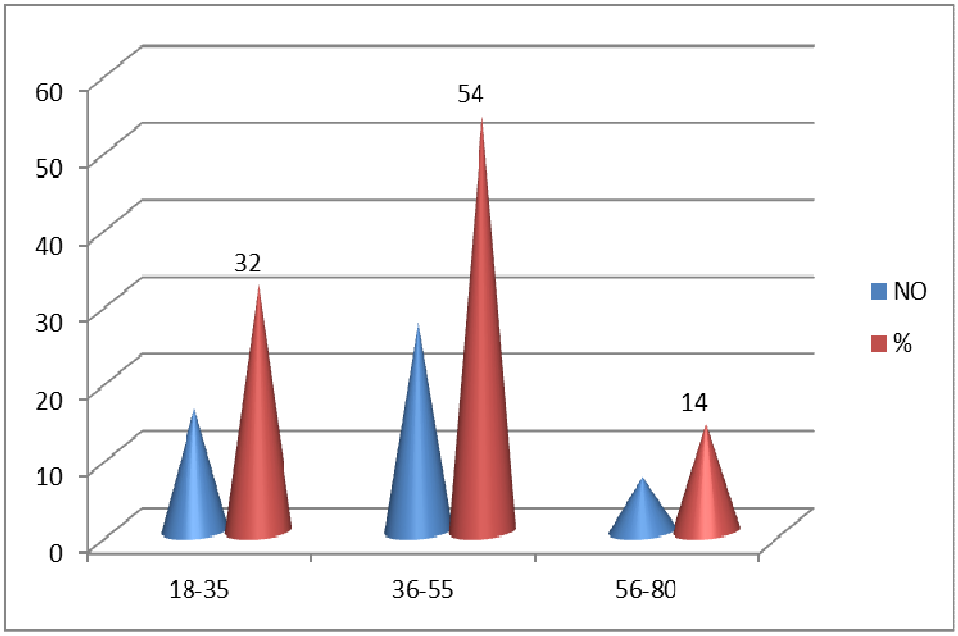
RESULTS

In total 50 confirmed DVT cases were recruited for the study. Blood Samples were collected and DNA was isolated and analyzed for the CYP2C9 *2, *3, *4 and *5.

Demographic features of study population

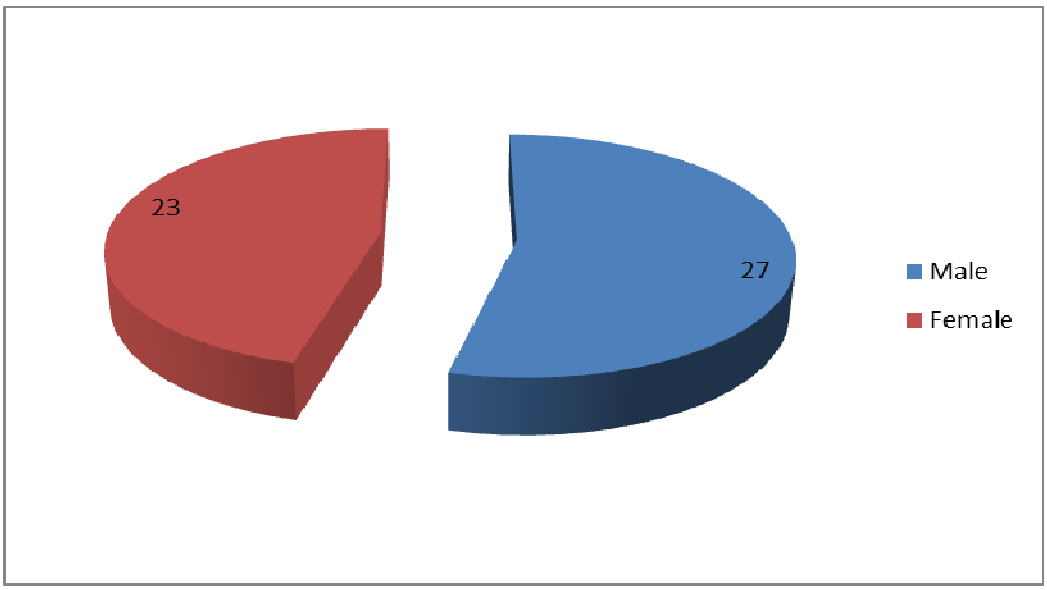
Age

Among the 50 cases of DVT selected in our study group, 32% were in the age group of 18-35 years and 54% were in the age group 36-55 years and 14% were in the age group of 56-80 years



Sex

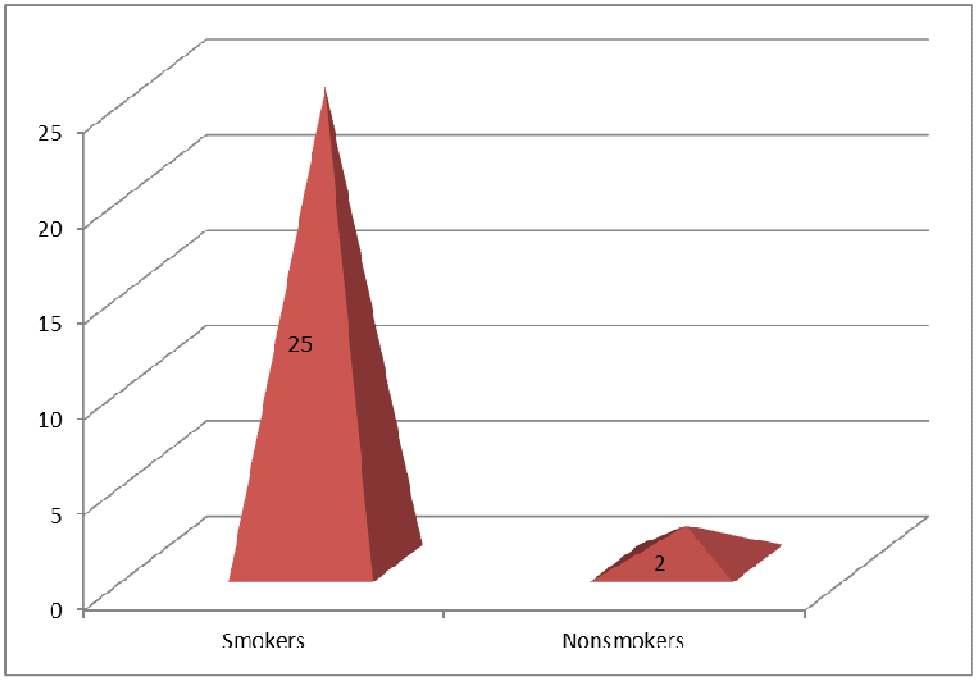
In this study group 54% of the cases were males and 46% were females. The incidence of the disease was seen more in the 36-60 years age group in both the sex.



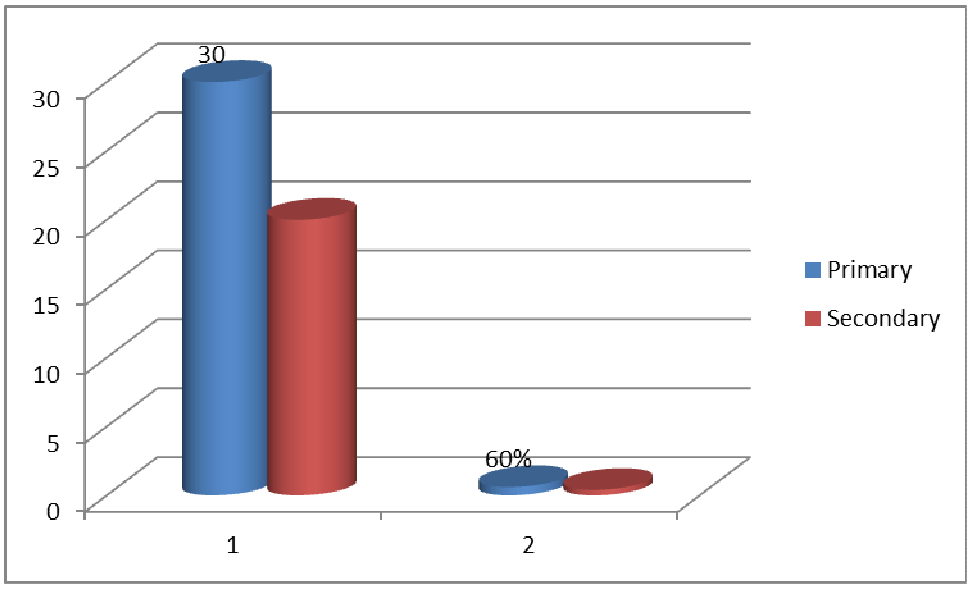
Distribution cases based on the sex

Smoking and Alcohol use

There was no history of smoking among females and 92% of males were smokers and among the smokers 52% of them were also alcoholic.

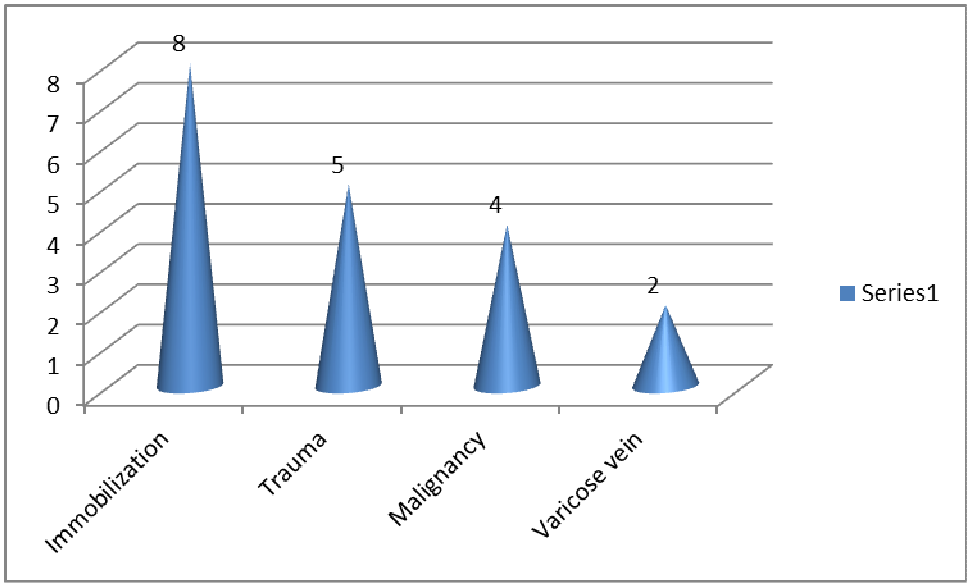


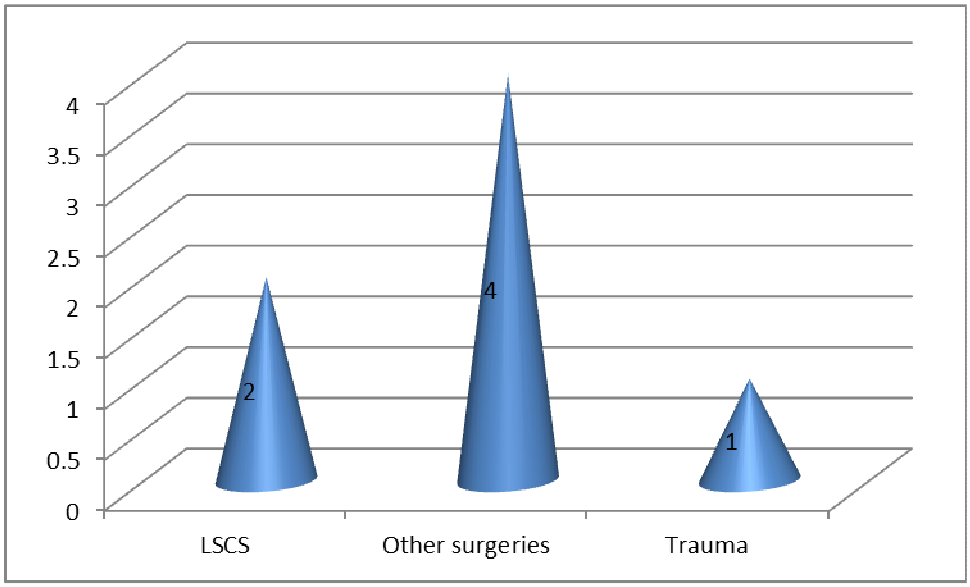
Smoking distribution in the DVT cases



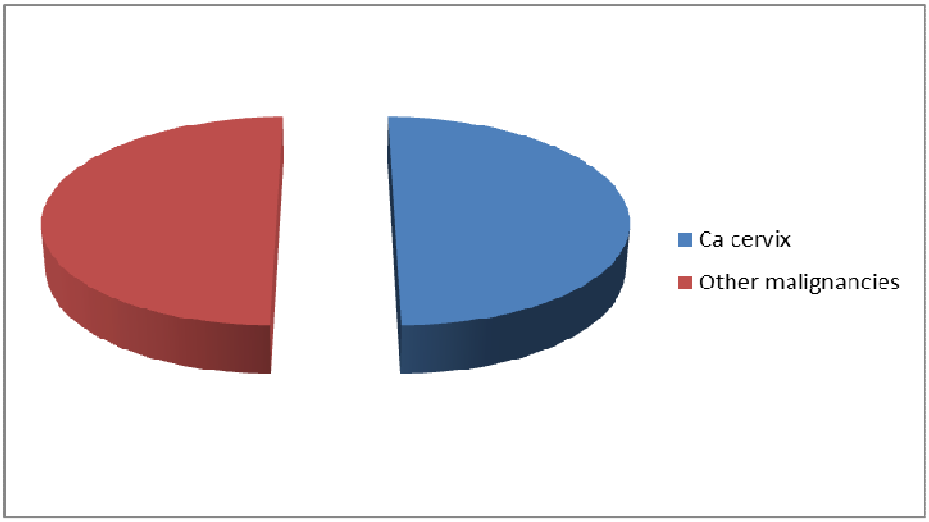
Etiological factors of the study group

Causes for DVT may be primary and secondary. Secondary causes includes trauma, immobilization, surgeris ,malignancies. Various etiologies are shown in the following



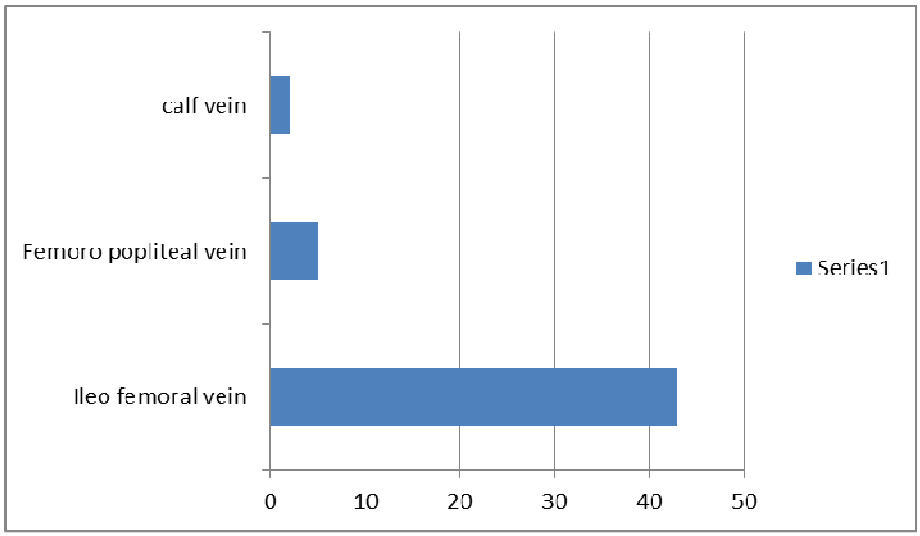


Secondary causes of the DVT cases



Secondary DVT due to malignancy

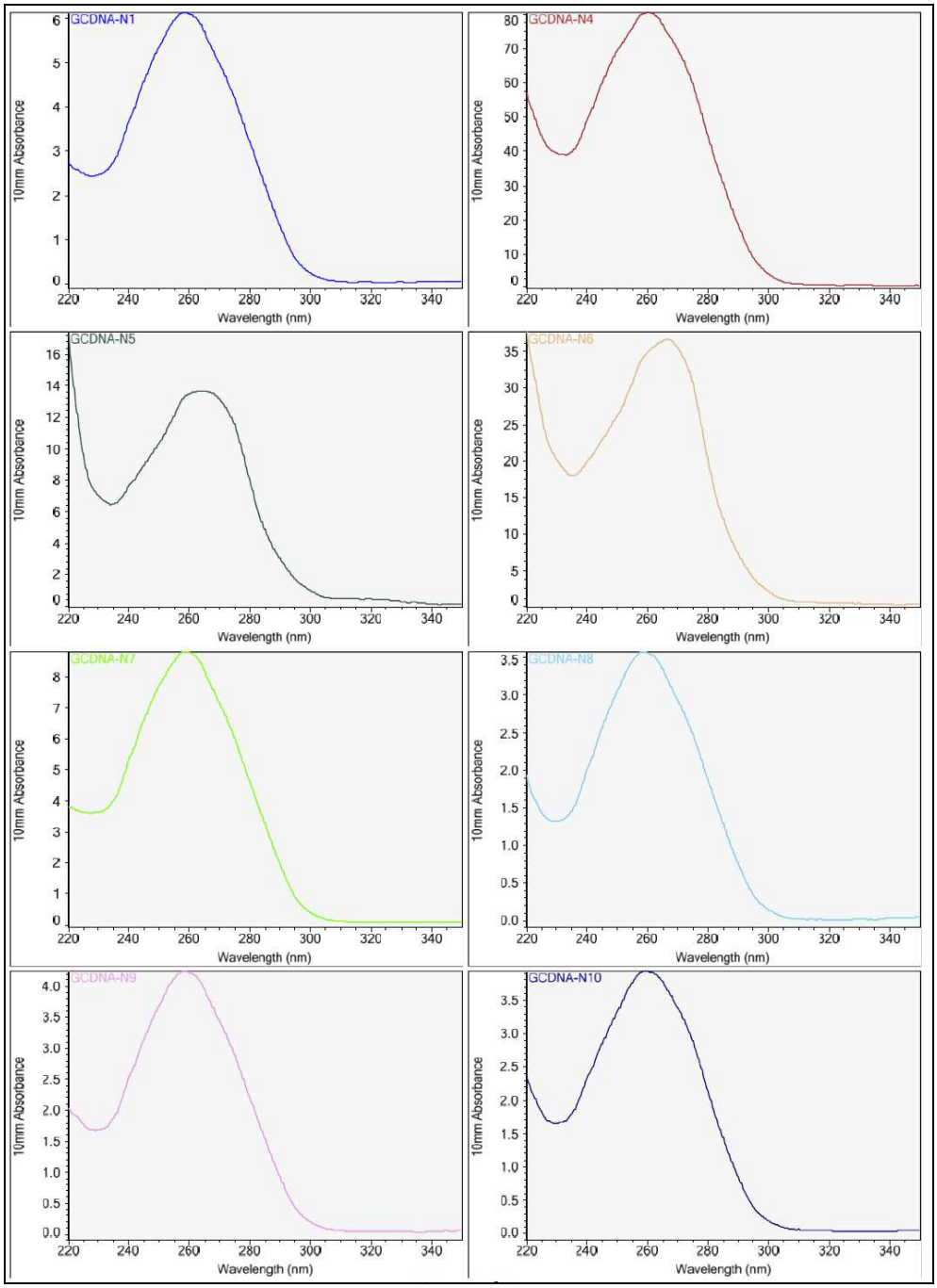
In our study group, 32 cases presented with left lower limb DVT and 18 cases presented with right lower limb DVT.



DNA isolation

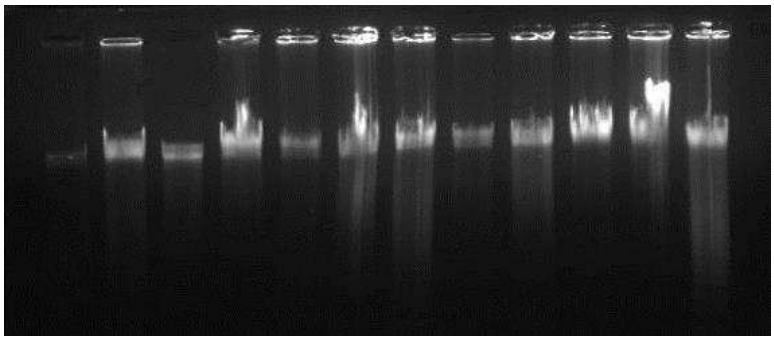
DNA was successfully isolated from all the DVT cases. The quantity and purity of DNA samples were found to be good (purity ranging from 1.8-2.0).

UV SPECTROMETER SCAN



Agarose gel electrophoresis of Genomic DNA

The quality of DNAs was checked in 1% Agarose gel running at 100 V for 30 min in 1X TAE buffer and it was found to be intact high molecular weight DNA.

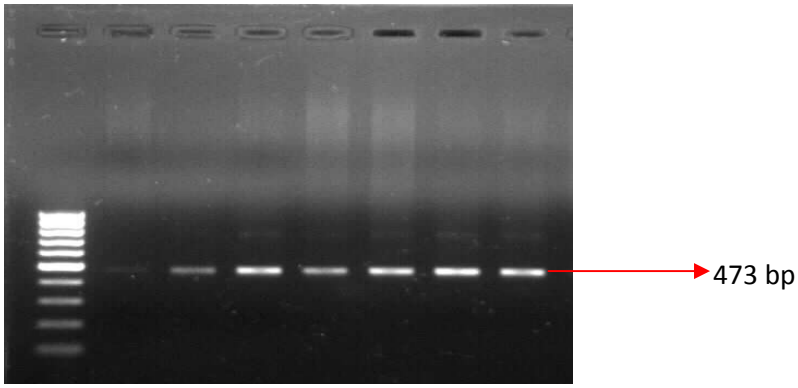


Genomic DNA Agarose Gel Electrophoresis (0.7% Agarose at 100 volts)

PCR amplification

CYP2C9 exon 1 and 4 were amplified with the sequence specific primers resulting in 473 bp and 256 bp respectively. Out of 50 samples 8 were not amplified in PCR and this may be due to presense of heparin in DNA preparation. Agarose gel electrophoresis of PCR amplified CYP2C9 exon 1 region covering the *2 variant (under the conditions 2% Agarose in 0.5X TAE at 100 Volts)

CYP2C9 PCR Exon 1 Product.

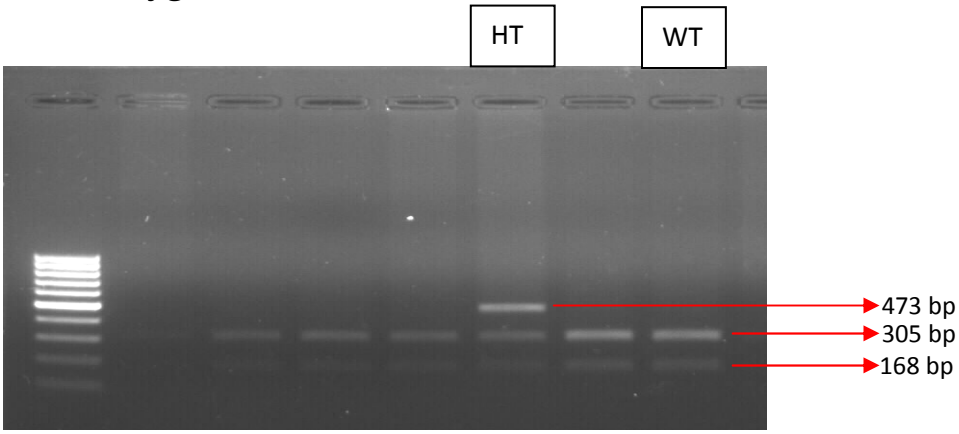


Restriction Fragment Length Polymorphism

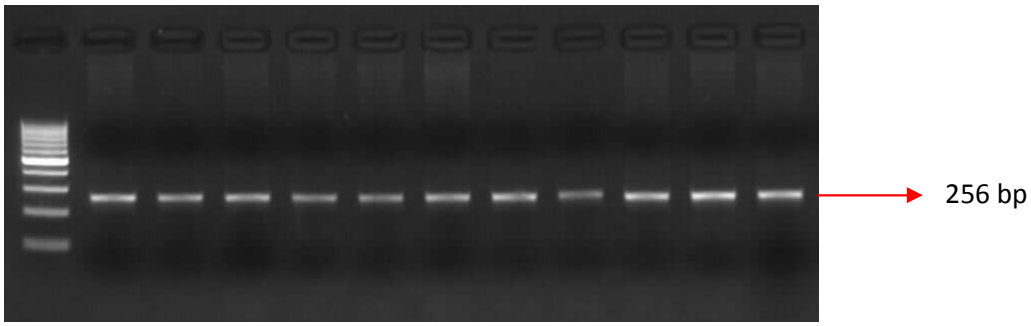
Expected digestion pattern of the CYP2C9*2 allele

Allele	CC (WT)	CT (HT)	TT
Digestion pattern (bp)		**473**	**473**
	305	**305**	
	168	**168**	

CYP2C9*2 variant was screened by PCR-RFLP using restriction enzyme *Sau 69I*. Out of 42 DNA samples 37 of them found to be wild type and only 5 cases were heterozygous for *2 mutation



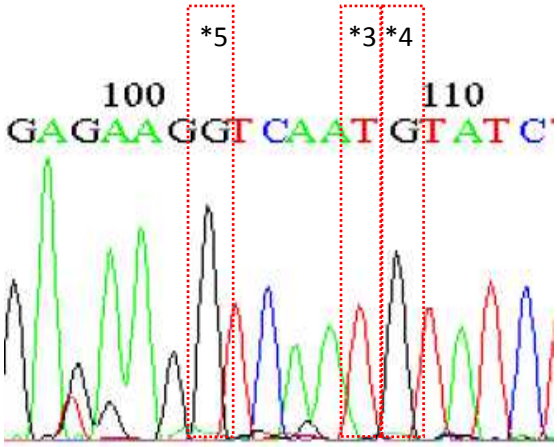
RFLP pattern of CYP2C9 exon 1 covering *2 variant. Agarose gel electrophoresis of *Sau 69I* digested CYP2C9 exon 2 (under the conditions 2% Agarose in 0.5X TAE at 100 Volts)



Agarose gel electrophoresis of PCR amplified CYP2C9 exon 4 region for *3, *4 and *5 variants (under the conditions 2% Agarose in 0.5X TAE at 100 Volts)

DNA sequencing

CYP2C9 *3 *4 *5 variants were identified by DNA sequencing of exon 4.Internal primer was used for sequencing to avoid interference from the family genes. All the 42 cases sequenced for *3,*4,*5 variants were found to be wild type (fig). In summary out of 42 cases analyzed only 5 were found to be heterozygous.

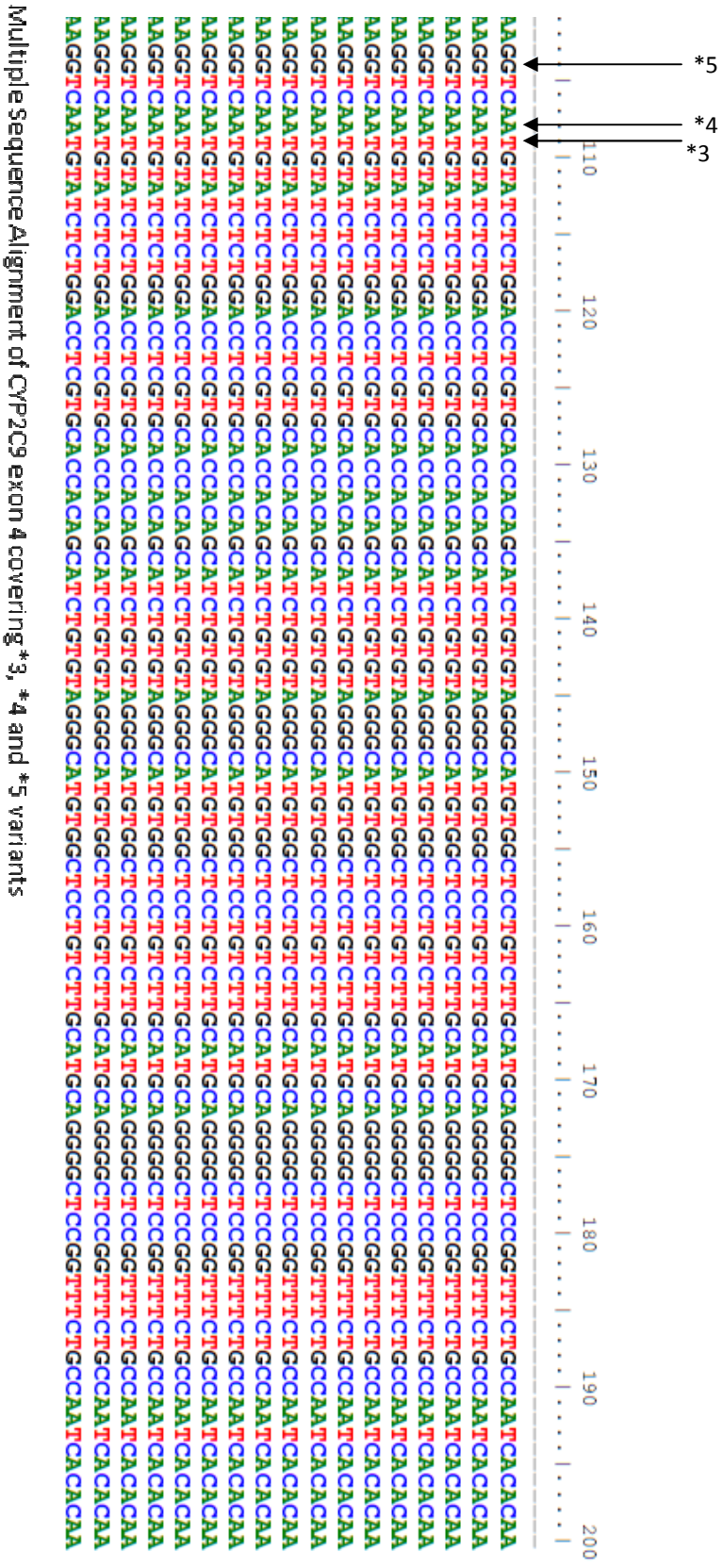


Sequencing Chromogram of CYP2C9 exon 4 showing wild type sequence for the *3, *4, and *5

Distribution of CYP2C9 *2,*3, *4, and *5 genetic variants in the current study population

	CYP2C9			
	rs1799853	rs1057910	rs1057909	rs28371686
Variants	C>T (*2)	A>C (*3)	T>C (*4)	C>G (*5)
Homozygous Wild	37 (88.1%)	42 (100%)	42 (100%)	42 (100%)
Heterozygous	5 (11.9%)	0 (0%)	0 (0%)	0 (0%)
Homozygous Mutant	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Multiple alignment of DNA sequences showing the wild type genotype of CYP2C9 *3,*4, and *5



DISCUSSION

Deep vein thrombosis occurs most often in the large veins of the legs such as the femoral vein or the popliteal vein and it is the one of the leading cause of morbidity and mortality. Deep vein thrombosis if not treated will /can lead to a life threatening pulmonary embolism. Warfarin is a hydroxyl coumarin derivative widely used as an anticoagulant for treating DVT patients with a narrow therapeutic index and wide inter-individual variability in dose requirement with the risk of thromboembolism or bleeding depending on underdosing or overdosing respectively.

Genetic variations in the VKORC1 and CYP2C9 have been shown to account for 30-50% of the variability in the required dosage of warfarin. Genetic variations of CYP2C9 are responsible for the pharmacokinetic effect on warfarin metabolism. Individuals with the CYP2C9*2 and CYP2C9*3 polymorphisms have decreased clearance of S-warfarin and therefore also an increased plasma concentration ratio when compared to R-warfarin. In patients with allelic variants the relative concentration of S-warfarin increases in patients homozygous for the CYP2C9*3 polymorphisms. The Genetic screening of VKORC1 and CYP2C9 polymorphisms may help in better and effective clinical management.

This pilot study was aimed to study the frequency of the CYP2C9*3 polymorphism rs1057910 along with associated polymorphisms (CYP2C9*3 (rs1057910), *4 (rs1057909) and *5 (rs28371686) in south in Indian DVT patients (n=50). Out of 50 DVT blood samples collected DNA isolation and sequencing was successful for 42 samples. CYP2C9*2 (rs1799853) variants was found in 5 patient samples in heterozygotes CT (11.9%) state. No homozygous mutant allele for CYP2C9*2 (rs1799853) was observed. The CYP2C9*2 (rs1799853) heterozygote may require less warfarin (in the medium range 4 mg). All the remaining patients with CYP2C9 wild type allele may be given the maximum dosage without the risk of bleeding.

SUMMARY AND CONCLUSION

1. Inter-individual variation in the effect of anticoagulants makes the treatment and management of DVT a challenging task.

2. Inappropriate dosing may results in adverse effects like under treatment or over treatment causing life threatening complication like bleeding.

3. Genetic variations in VKORC1 and CYP2C9 were shown to influence the dose requirement of Warfarin.

4. Fifty Deep vein thrombosis patients with primary and secondary DVT were recruited for the study with varying age group and appropriate exclusion criteria.

5. CYP2C9 genetic variants *2, *3, *4 and *5 were analyzed in 42 cases successfully. Out of 42 samples analyzed 5 samples were found to be heterozygous for CYP2C9 *2 variant.

6. All other cases were found to carry wild type alleles for all the genetic variants analyzed in this study.

7. The cases having CYP2C9 *2 heterozygous variant require decreased dose of warfarin by approximately 20-30% compared to CYP2C9 wild type individuals.

LIMITATIONS OF THE STUDY

Testing with more number of samples will identify the frequency of these CYP2C9 genetic variants *2, *3, *4 and *5 in south Indian population and may help in better and effective clinical management. This study should be extended to analyze the associated VKORC1 genetic variants and to suggest the appropriate dosage based on the genetic study.

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ABBREVIATIONS

DVT	Deep vein thrombosis
PE	Pulmonary embolism
VTE	Venous thromboembolism
HIF-1	Hypoxia inducible factor-1
ELISA	Enzyme linked immunosorbent assay
SLE	Systemic lupus erythematosus
HIT	Heparin induced thrombocytopenia
AT	Antithrombin
RE SYSTEM	Reticulo endothelial system
VKORC1	Vitamin K epoxy reductase complex
PT	Prothrombin time
INR	International Normalized Ratio
ICH	Intra cerebral haemorrhage
LMWH	Low molecular weight heparin

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3
Telephone No : 044 25305301
Fax : 044 25363970
EC RegNo.ECR/270/Inst./TN/2013

CERTIFICATE OF APPROVAL

To
Dr.M.Selvi,
MD General Medicine PG,
Madras Medical College, Chennai-3.

Dear M.Selvi,

The Institutional Ethics committee of Madras Medical College, reviewed and discussed your application for approval of the proposal entitled "Screening of CYP4502C9 Genetic Polymorphism Associated with Acenocoumarol Sensitivity in DVT Cases " No.17062013.

The following members of Ethics Committee were present in the meeting held on 11.06.2013 conducted at Madras Medical College, Chennai -3.

1. Dr.SivaKumar, MS FICS FAIS --- Chairperson
2. Prof. R. Nandhini MD -- Member Secretary
   Director, Instt. of Pharmacology ,MMC, Ch-3
3. Prof. Shyamraj MD -- Member
   Director i/c , Instt. of Biochemistry , MMC, Ch-3
4. Prof. P. Karkuzhali. MD -- Member
   Prof., Instt. of Pathology, MMC, Ch-3
5. Prof. A. Radhakrishnan MD -- Member
   Prof of Internal Medicine, MMC, Ch-3
6. Prof. S. Deivanayagam MS -- Member
   Prof of Surgery, MMC, Ch-3
7. Thiru. S. Govindsamy. BABL -- Lawyer
8. Tmt. Arnold Saulina MA MSW -- Social Scientist

We approve the proposal to be conducted in its presented form.

Sd/ Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, and SAE occurring in the course of the study, any changes in the protocol and patients information / informed consent and asks to be provided a copy of the final report.

R Nadini 1/7/13

Member Secretary, Ethics Committee

S.No	IP.No		Name of Patien	Age	Sex	Smoking/A	DVT Side	Level	Etiology	Risk Factor	Duration of Symptoms
DVT1		8350	Annammal	60	F	-	Right	I liofemoral	Secondary	Cervical Cancer Stage III / imn	1 month
DVT2		9289	Kaleem	34	M	S/A	Left	I liofemoral	Primary	-	15 days
DVT3		9599	Lakshmi	47	F	-	Right	Femoropopliteal	Secondary	CaCx	5 days
DVT4		10606	Mahesh	65	M	S	Right	I liofemoral	Primary	Suspected Malignancy, Gener	1 week
DVT5		10623	Balakrishnan	65	M	S	Left	I liofemoral	Primary	-	1.5 month
DVT6		10847	Mangaiyarkarasi	55	F	-	Left	I liofemoral	Primary	-	1 week
DVT7		13328	Shak Abbas	28	M	S/A	Right	I liofemoral	Primary	-	5 days
DVT8		13390	Lakshmi	42	F	-	Left	I liofemoral	Primary	-	1 month
DVT9		17038	Thirupathy	40	M	S/A	Right	I liofemoral	Primary	-	1 week
DVT10		16787	Mahendran	55	M	S	Left	I liofemoral	Primary		20 days
DVT11		15963	Govindan	70	M	S/A	Left	I liofemoral	Primary		2 months
DVT12		17026	Manivannan	35	M	S/A	Left	Femoropopliteal	Primary	-	10 days
DVT13			Palani	45	M	S	Right	I liofemoral	Secondary	Trauma	10 days
DVT14		20873	Govindaraj	55	M	S	Left	I liofemoral	Secondary	Malignancy	4 days
DVT15		18669	Rajan	45	M	S	Right	Calf Vein	Primary	Swelling in legs	1 month
DVT16		18677	Kala	50	F	-	Right	I liofemoral	Secondary	Malignancy	10 days
DVT17		18694	Murugan	27	M	S	Right	I liofemoral	Primary	Immobolization	2 weeks R/ 4 months L
DVT18		19667	Rajammal	50	F	-	Left	I liofemoral	Primary	Swelling in legs	15 days
DVT19		21170	Valarmathy	36	F	-	Left	I liofemoral	Primary	Fioroid Utereus	4 days
DVT20		28786	Mahalaxmi	42	F	-	Left	I liofemoral	Secondary	Heptoectomy	10 days
DVT21	1896-10		Kalaivani	20	F	-	Left	Calf Vein	Secondary	Trauma	
DVT22		42036	Shanthi	40	F	-	Left		Secondary	Varicose veins	
DVT23		41071	Raguvaran	29	M	S/A	Left	I liofemoral	Primary	-	2 weeks
DVT24		41854	Krishnamoorthy	53	M	S/A	Left	Femoropopliteal	Primary	-	10 days
DVT25		40652	Mani	42	M	S	Left	I liofemoral	Secondary	Trauma	5 days
DVT26		37959	Rathinaraj	45	M	S/A	Left	I liofemoral	Primary	Trauma, immobolization	2 weeks
DVT27		35841	Selvi	23	F	N	Left	I liofemoral	Secondary	LSCS	10 days
DVT28		35788	Lakshmi	35	F	-	Left	I liofemoral	Primary	-	2 weeks
DVT29		43663	Vasantha	55	F	-	Left	I liofemoral	Secondary	Trauma, immobolization	15 days
DVT30		43106	Gopalakrishnan	60	M	S	Right	I liofemoral	Primary	-	10 days
DVT31		37290	Maheswari	40	F	-	Left	I liofemoral	Primary	-	10 days

DVT32	59494	Guna	29	F	-	Left	I liofemoral	Secondary	immobolization	10 days
DVT33	59517	Prabhakaran	38	M	S	Left		Primary	-	5 days
DVT34	58164	Malarvizhi	23	F	-	Right	I liofemoral	Secondary	Varicose veins	7 days
DVT35	53254	Nateesa	27	F	-	Bilateral	I liofemoral	Secondary	immobolization	2 days L / 1 month R
DVT36	57718	Ravichandran	46	M	S/A	Right	I liofemoral	Primary	-	2 weeks
DVT37	59461	Velankanni	35	M	S/A	Left	I liofemoral	Primary	-	1 month
DVT38	57098	Murugananthan	42	M	S/A	Left	I liofemoral	Primary	-	10 days
DVT39	54902	Sumathy	19	F	-	Left	I liofemoral	Secondary	immobolization, Post operativ	5 days
DVT40	62426	Sanjeev Reddy	51	M	S	Right	I liofemoral	Primary	-	1 week
DVT41	62615	Ramya	23	F	-	Left	I liofemoral	Secondary	LSCS	1 week
DVT42	63079	Chinnadurai	21	M	S/A	Left	Femoropopliteal	Primary	-	5 days
DVT43	67410	Saamanthi	86	F						
DVT44	65402	Saroja	60	F						
DVT45	65675	Shyamala	42	F						
DVT46	62656	Sekar	40	M						
DVT47	67514	Arumugam	43	M	S/A	Left	I liofemoral	Primary	-	5 days
DVT48	67823	Kavitha	31	F	-	Left	Femoropopliteal	Primary	-	5 days
DVT49	68732	Mohammed Abc	39	M	-	Right	I liofemoral	Primary	-	2 weeks
DVT50	69018	Selvam	44	M	S/A	Left	I liofemoral	Secondary	Trauma	10 days

INFORMATION SHEET

We are conducting a study on **"SCREENING OF CYP450 2C9 GENETIC POLYMORPHISM ASSOCIATED WITH ACENOCOUMAROL SENSITIVITY IN DVT CASES"** among patients attending Rajiv Gandhi Government General Hospital, Chennai and for that your specimen may be valuable to us.

The purpose of this study is to screen the genetic polymorphism of the acenocoumarol metabolizing enzyme *CYP450 2C9*.

We are selecting certain cases and if you are found eligible, we may be using your specimen to perform studies which in any way do not affect your final report or management.

The privacy of the patients in the research will be maintained throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

Taking part in this study is voluntary. You are free to decide whether to participate in this study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.

The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Signature of Investigator | Signature of Participant

Date :
Place :

PATIENT CONSENT FORM

Study Detail	:	**SCREENING OF CYP450 2C9 GENETIC POLYMORPHISM ASSOCIATED WITH ACENOCOUMAROL SENSITIVITY IN DVT CASES**
Study Centre	:	Rajiv Gandhi Government General Hospital, Chennai.
Patient's Name	:	
Patient's Age	:	
Identification Number	:	

Patient may check (☑) these boxes

I confirm that I have understood the purpose of procedure for the above study. I have the opportunity to ask question and all my questions and doubts have been answered to my complete satisfaction. ☐

I understand that my participation in the study is voluntary and that I am free to withdraw at any time without giving reason, without my legal rights being affected. ☐

I understand that sponsor of the clinical study, others working on the sponsor's behalf, the ethical committee and the regulatory authorities will not need my permission to look at my health records, both in respect of current study and any further research that may be conducted in relation to it, even if I withdraw from the study I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published, unless as required under the law. I agree not to restrict the use of any data or results that arise from this study. ☐

I agree to take part in the above study and to comply with the instructions given during the study and faithfully cooperate with the study team and to immediately inform the study staff if I suffer from any deterioration in my health or well-being or any unexpected or unusual symptoms. ☐

I hereby consent to participate in this study. ☐

I hereby give permission to undergo complete clinical examination and diagnostic tests including hematological, biochemical, radiological tests. ☐

Signature/thumb impression	Signature of Investigator
Patient's Name and Address:	Study Investigator's Name: **Dr. M. SELVI**

DVT clinical data sheet and Patient consent

Hospital IP No: Date:

Lab ID:

Name: Age: Sex:

Address:

Occupation:

Smoking: Alcohol:

Patient History:

Clinical Details:

DVT-Side: R/L

Level: I liofemoral/Femoropopliteal/Calf vein

Etiology-Primary/Secodary

Assoc Risk factors of Provoked DVT: Malignancy/trauma/immobolization/varicose veins/thrombophlebitis/CVA/CCF

Duration of symptoms

Assoc Symptomatic PE

Comorbid conditions

Treatment history and details

Rheumatology/Hematology findings

Investigations:

CBC

RFT

ECG

CXR

BT/CT/PT/APTT

Duplex findings

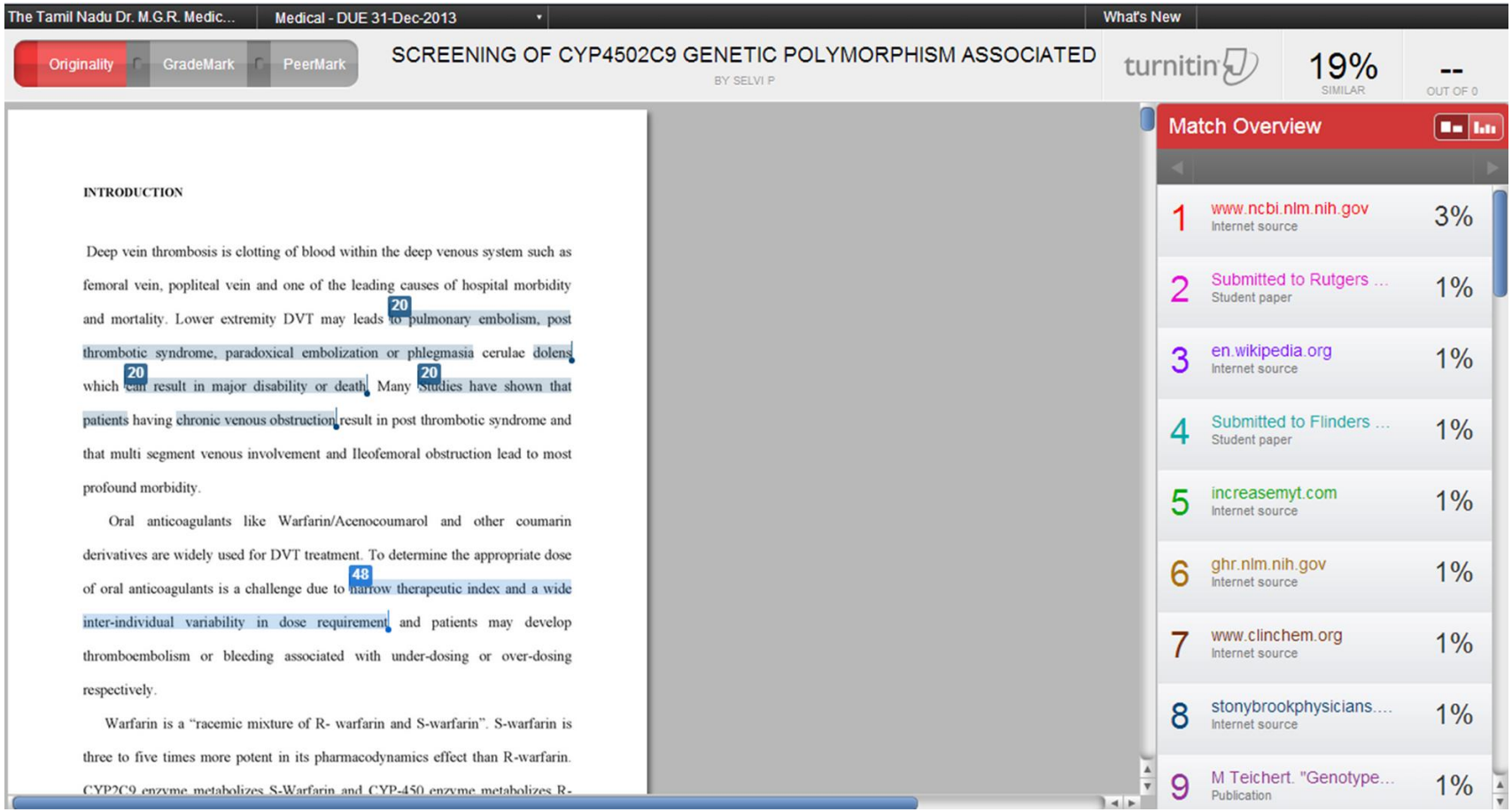
Treatment History:

Hypertension:

Diabetics History:

Cardio Vascular Disease History:

Family History: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INTRODUCTION
Deep vein thrombosis is clotting of blood within the deep venous system such as femoral vein, popliteal vein and one of the leading causes of hospital morbidity and mortality. Lower extremity DVT may leads to pulmonary embolism, post thrombotic syndrome, paradoxical embolization or phlegmasia cerulae dolens which can result in major disability or death. Many studies have shown that patients having chronic venous obstruction result in post thrombotic syndrome and that multi segment venous involvement and Ileofemoral obstruction lead to most profound morbidity.
Oral anticoagulants like Warfarin/Acenocoumarol and other coumarin derivatives are widely used for DVT treatment. To determine the appropriate dose of oral anticoagulants is a challenge due to narrow therapeutic index and a wide inter-individual variability in dose requirement and patients may develop thromboembolism or bleeding associated with under-dosing or over-dosing respectively.
Warfarin is a "racemic mixture of R- warfarin and S-warfarin". S-warfarin is three to five times more potent in its pharmacodynamics effect than R-warfarin. CYP2C9 enzyme metabolizes S-Warfarin and CYP-450 enzyme metabolizes R-
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INTRODUCTION Deep vein thrombosis is clotting of blood within the deep venous system such as femoral vein, popliteal vein and one of the leading causes of hospital morbidity and mortality. Lower extremity DVT may leads to pulmonary embolism, post thrombotic syndrome, paradoxical embolization or phlegmasia cerulae dolens which can result in major disability or death. Many Studies have shown that patients having chronic venous obstruction result in post thrombotic syndrome and that multi segment venous involvement and lleofemoral obstruction lead to most profound morbidity. Oral anticoagulants like Warfarin/Acenocoumarol and other coumarin derivatives are widely used for DVT treatment. To...